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A LABORATORY GUIDE

IN

PRACTICAL BACTERIOLOGY

WITH AN OUTLINE FOR THE CLINICAL EXAMINATION  
OF THE URINE, BLOOD AND GASTRIC CONTENTS,

BY

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THIRD EDITION, REVISED.

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## PREFACE TO THE THIRD EDITION.

This book presents in more permanent form the outlines of work covered in my practical classes in Bacteriology and Clinical Microscopy. No more detail has been included than experience has shown to be requisite.

In this edition such alterations and additions have been incorporated as will bring the methods in accord with our present laboratory practice.

W. T. CONNELL.

July 1st, 1913.

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## PART I.

### PRACTICAL BACTERIOLOGY.

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#### LABORATORY RULES.

As students will have to work with many of the actual causative factors of diseases, viz., the pathogenic bacteria, there is a liability to self-infection or infection of other workers unless care is taken to develop a proper technique. The practical work is started with non-pathogenic species, or with those of low pathogenic power, so that such a technique may be acquired without danger. The observance of the following general rules is essential:

1. All possible cleanliness should be observed in the care of desk, lockers, and apparatus. To this end all glassware should be cleaned immediately after use and returned to its place.

2. If infectious matter should by accident come in contact with the hands, or fall on table or floor, 1/1000 bichloride solution should be freely and immediately employed.

3. All soiled material, scraps or any infected matter, should be thrown into crocks provided for that purpose and not into the sinks.

4. The platinum needles used in making and transferring cultures should be sterilized in the flame shortly before and immediately after use.

5. At close of work, all apparatus should be returned to its place and gas turned out. Before leaving laboratory hands should be washed well with soap and water, then in 1/1000 bichloride, rinsing finally with water.







## LESSON I.

Inoculation of culture media from pure cultures of Bacteria—  
Bacteriological microscope.

Students before beginning work should familiarize themselves with the characters of the common culture media; the method of holding culture tubes and removing and replacing plugs; the sterilization and care of the platinum needle or loop; and the labelling and incubating of the inoculated tubes. In making inoculations very *minute* amounts of the growth suffice to be transferred. The culture tubes, after inoculation, should be carefully labelled with the name of the organism, the date and name of student. Drawings will be found valuable in keeping records of the morphological and cultural characters of the bacteria studied.

1. Make two agar slope cultures of *Bacillus prodigiosus*.

Place one tube in the incubator at 37°C. (98.6°F.), and the other at 21°C. (70°F.).

Examine these tubes at the end of 24 and 48 hours. Growth is more rapid at the higher temperature, but the pigmentation is slight, while a more slowly appearing growth, deeply pigmented, is noted in the other tube.

Pigmentation is in many chromogenic species, more marked at the lower temperature than at the higher.

2. Make agar slope cultures of (a) *Bacillus cyanogenus*, (b) *Bacillus pyocyaneus*, and place in the incubator at 21°C.

Examine each day and note the gradual intensification of the pigmentation of the media, the growths being largely uncolored.

3. Make gelatine stab cultures of (a) *Staphylococcus pyogenes aureus*, (b) *Bacillus fluorescens liquefaciens*.

Incubate at 21°C. and examine daily. These bacteria liquefy gelatine more or less rapidly.

4. Make potato cultures of (a) *Sarcina lutea*, (b) *Bacillus ruber*, (c) *Torula rosea*. Incubate at 21°C. and examine daily, noting development of pigment.

5. Make cultures on coagulated blood serum of (a) *Bacillus proteus vulgaris*, (b) *Spirillum milleri*. Incubate at 37°C. and examine daily. These bacteria liquefy the coagulated serum.

6. Make broth cultures of (a) *Bacillus subtilis*, (b) *Streptococcus pyogenes*, (c) *Staphylococcus pyogenes albus*. Incubate at 37°C. and examine daily, noting such points as scum, turbidity and sediment.





7. Make litmus milk cultures of (a) *Bacillus acidilactici* (Esten), (b) *Bacillus lactis aerogenes*, (c) *Bacillus lactis viscosus*. Incubate the two former at 37°C., latter at 21°C. Note such points as acid production, curdling, gas formation and viscosity.

8. Make agar slope cultures of (a) *Staphylococcus pyogenes aureus*, (b) *Spirillum milleri*.

*On use of Microscope.*

The microscope employed for bacteriological examination should have as minimum requirements an Abbé condenser, triple nose piece, two oculars, low and high power dry objectives and an oil-immersion objective. The dry objectives are the same as are used in normal and morbid Histology, the low power objective usually having a focal distance of  $\frac{2}{3}$ " (16 mm.), the high power of  $\frac{1}{6}$  or  $\frac{1}{7}$ " (4 mm.). The front lenses of these objectives must be kept dry and material to be examined may either be suspended in water or other liquid, (the lens being protected when necessary by interposition of a cover glass,) or mounted in Canada balsam or like medium. The oil-immersion objective has usually a focal distance of  $\frac{1}{12}$ " (2 mm.). In use the front lens is immersed in a special oil having the same refractive index as glass. The oil may be placed directly on film to be examined if this be dry, or on top of cover glass if the film be mounted. Very thin (No. 1) cover glasses are required in using this objective.

In the microscopical examination of an unstained object (structure image) the object is seen only as it obstructs the rays of light passing from the mirror to the lens and thence to the eye. The light must thus be carefully adjusted to the opacity of the object to be examined. The clearer it is, the less light will be required for its examination, a full flood of light causing such clear objects to disappear. Care must thus be taken in the examination of unstained objects to so moderate the light by closure of the diaphragm as to give the sharpest shadow or structure image. Bacteria when unstained present but slight obstruction to the rays of light and some are not rendered visible even with such moderation of light especially when examined in body fluids. In such cases "dark-ground" illumination (Lesson IV) may be used, or the bacterium may be stained. The stain increases the opacity of the object so that it stands out in relief against a flood of light (color image). Further, the intensity and character of staining assist materially in bringing out structural details.

The Abbé condenser, while not required for low and high powers, is essential when using the oil-immersion, to concentrate the light upon the object and thence into the small aperture of this objective.

#### *Use of oil immersion objective.*

9. Stained preparations of *Staphylococcus pyogenes aureus*, *Bacillus anthracis* and *Spirillum cho-*







lerae asiaticae are provided. Examine these as follows: Put a drop of immersion oil on the centre of the film to be examined, and placing the preparation on the centre of the microscope stage, fix one end of the slide with a clip. Lower the lens with the coarse adjustment, till it touches the drop. With the eye to the ocular, lower very slowly till the field becomes colored. Now, with the fine adjustment carefully lower the lens till the field comes clearly into view. The slide can be readily moved about at the unclipped end. Before removing slide, always raise the objective well out of the oil with the coarse adjustment.

On completing day's work, remove oil from lens by use of a soft cloth or with lens paper. Saliva will be found very efficacious in assisting to clear a smeared lens. Avoid the use of xylol, ether or alcohol in cleansing, as these reagents dissolve the embedding substance of lens.

## LESSON II.

Stains—Staining and examination of pure cultures of bacteria from solid media.

*Stains and general principles of staining.*

The stains used in Bacteriology are certain of the anilin dyes and may be grouped into two main classes, basic and acid. As a class the basic dyes stain bacteria and the nuclei of tissue cells, while acid dyes stain tissue protoplasm. The basic dyes in common use are methylene blue, gentian violet, and fuchsin, and are usually employed in aqueous dilutions of the saturated alcoholic solutions. Eosin is the common acid dye and is used either in diluted alcoholic or aqueous solution. The staining capacity or combining power of the dyes may be increased by heating or by addition of certain chemicals, for instance anilin oil, carbolic acid or dilute alkalies. Again during or after staining, the stain may be modified, differentiated or removed by use of water, alcohol, acids, iodine solutions or other chemicals. Throughout the practical work examples will be found of adoption of these in staining.

Combined staining or counter-staining is frequently employed when two or more structures are present which react differently towards stains.

The staining solutions ordinarily used are:





(a) Methylene blue. This may be used in several forms. The stain most commonly employed is Loef-  
fler's alkaline solution. Its composition is,

Saturated alcoholic solution of methylene  
blue . . . . . 30 cc.  
1 in 10000 caustic potash solution . . . 100 cc.

Methylene blue may also be employed as a 2% aqueous solution of the dye, or as carbol methylene blue, consisting of 10 cc. of the saturated alcoholic solution and 90 cc. of a 5% aqueous solution of carbolic acid.

(b) Gentian violet. This stain is used in several forms. Of these anilin gentian violet is most used. It consists of,

Anilin oil . . . . . 4 cc.  
Distilled water . . . . . 100 cc.

Shake thoroughly, filter and add saturated alcoholic solution of gentian violet . . . . . 25 cc.  
This stain ought to be freshly prepared every two or three weeks.

Gentian violet may also be employed in aqueous solution, made by adding 2 cc. of filtered saturated alcoholic solution of the dye to 100 cc. of water. In this form it proves very satisfactory for pure culture staining. Carbol gentian violet may be also employed and is made like the corresponding methylene blue stain.

(c) Fuchsin. Several staining solutions are used of this dye, of these Ziehl's carbol-fuchsin is most useful. This stain is made by taking

Saturated alcoholic solution of fuchsin	
crystals . . . . .	10 cc.
5% carbolic acid solution . . . . .	90 cc.

Fuchsin can also be used as anilin water fuchsin, or as diluted fuchsin.

(d) Eosin. This is employed either as 1 in 1000 aqueous solution or as alcoholic eosin, a .5% solution in 70% alcohol. For the former, yellow water soluble eosin is used, for the latter alcoholic eosin.

The composition of other stains may be found by reference to Index.

*All stains must be filtered before use.*

Cover glasses should be kept in alcohol and handled with forceps. When they are required for use, rub dry with a clean cloth. Slides are preferable to cover glasses for ordinary work and should be kept and handled in a manner similar to the cover glasses.

Cultures especially of the rapidly growing bacteria are best examined when from one to three days old. After this period involution forms frequently develop.

*Examination of cultures from solid media.*

1. (a) Take a clean cover glass or slide and sterilize by passing it quickly through the gas (or spirit lamp) flame. Then with a sterilized platinum loop







place a drop of sterile water on cover glass or slide. Remove then with due precautions a *minute* amount of the agar culture of *Bacillus prodigiosus* and smear this with the water evenly over the surface of the cover glass, or corresponding area of a slide.

(b) Allow the film to dry in the air or by holding it high over the flame. Fix the film if a cover glass by passing it *quickly* three times through the flame, *slowly* if on slide.

(c) Cover the smeared surface of the slide or the cover glass with anilin gentian violet, for one to two minutes. If desired the cover glass can be floated film surface down on the stain.

(d) Remove and wash in water. If it is not desired to preserve specimen the cover glass may, after washing, be mounted in a drop of water, taking care to remove all water from upper surface of the glass before examination. If the cover glass or slide is to be preserved the smear should be dried thoroughly by use of blotting pad and gentle heat and then mounted in Canada balsam. Ordinarily with smear on slide, the immersion oil is placed directly on the dried film surface when being examined.

Examine the specimen with the oil immersion objective, using the plane mirror and open diaphragm, with the Abbé condenser.

2. In like manner prepare and stain smears from the agar cultures of *Bacillus cyanogenus* and *Bacillus pyocyaneus*.

3. Prepare smears from the potato cultures of *Torula rosea*, *Sarcina lutea* and *Bacillus ruber*. Stain with Loeffler's methylene blue for one to two minutes.

4. Prepare smears from the cultures of *Spirillum milleri* and *Staphylococcus pyogenes aureus*. Stain with carbol-fuchsin diluted three times with water, leaving in stain one-half minute.

5. Make broth cultures of *Staphylococcus pyogenes aureus*, *Streptococcus pyogenes*, *Bacillus pyocyaneus*, *Bacillus subtilis* and *Spirillum milleri* for use in Lesson III. Incubate at 37°C. for 24 hours.





## LESSON III.

Examination of pure cultures of bacteria grown on liquid media—Gram's method of staining.

1. After shaking the tube, to get an even admixture of the contained bacteria, with a sterilized platinum loop place a drop of the broth culture of *Staphylococcus pyogenes aureus* on slide (or cover glass) and spread into a thin smear. Dry and fix by passing three times through the flame and then place in 10% acetic acid for 5 minutes to clear away any of the media which might adhere to the bacterial cells. Wash in water and then cover with Loeffler's methylene blue for one to two minutes. Again wash in water, dry and examine.

2. Examine in like manner the broth cultures of *Streptococcus pyogenes* and *Bacillus subtilis*. Use Loeffler's methylene blue for staining.

3. In like manner examine the broth cultures of *Bacillus pyocyaneus* and *Spirillum milleri*. Use anilin gentian violet for staining.

*Gram's method.*

Gram's method of staining is based on the fact that, when some bacteria are stained with certain dyes (usually anilin or carbol gentian violet), and then treated with a solution of iodine, the iodine fixes the stain so that it is not washed out by after treat-

ment with alcohol. It affords a good differential stain, as some species stain, while others do not. Various modifications will be noted in this method, for the staining of pus and tissues.

4. Prepare slide or cover glass smears from the broth and from the agar cultures of *Streptococcus pyogenes* and *Staphylococcus pyogenes aureus*. Dry and fix in the usual manner. Stain them according to Gram's method as follows:

(a) Place the films in alcohol one to two minutes.

(b) Without drying, transfer to anilin gentian violet for three minutes.

(c) Rinse in water, and then place for three minutes in Gram's iodine solution. (Iodine 1 part, Potassium iodide 2 parts, Water 300 parts).

(d) Rinse in water, and then in alcohol, till no more stain comes away.

(e) Wash in water, dry and examine.

5. In like manner prepare smears from the broth cultures of *Bacillus subtilis*, *Bacillus pyocyaneus* and *Spirillum milleri*. Stain by Gram's method and note whether positive or negative.

6. Make broth cultures of (a) *Staphylococcus pyogenes aureus*, (b) *Bacillus proteus vulgaris*, (c) *Spirillum milleri*. Incubate at 37°C. for 24 hours.







## LESSON IV.

Examination of bacteria unstained—Hanging drops—Examination by dark ground illumination.

1. After shaking the tube, remove with a sterile loop a drop of the broth culture of *Staphylococcus pyogenes aureus*, and place on a cover glass. Invert this cover glass on a slide.

Examine first with the high dry power with diaphragm nearly closed and then with the oil immersion lens, slightly opening the diaphragm.

2. In like manner examine the broth cultures of *Bacillus proteus vulgaris* and *Spirillum milleri*. These bacteria show marked motility.

Note apparent size of these bacteria and contrast with the stained preparations made in preceding lesson.

3. Examine in the same manner a drop of putrefying urine (48 hours old). Large numbers of bacteria, usually of several species, are seen. Motile forms are common.

4. In like manner examine a drop of hay infusion which has been permitted to stand in the incubator at 37°C. for 24 hours or at room heat for several days. Note bacterial and other plant and animal forms present.

*Hanging Drops.*

The motility of bacteria is usually studied in hanging drop preparations. Under the microscope we may make out three kinds of movement: (a) Current movement, where the bacteria move with the suspending fluid. This movement will be well seen in some of the specimens already prepared. (b) Molecular or "Brownian" movement, where the bacteria vibrate in the fluid, as do all solid particles suspended in fluids. In this movement there is no actual change in position of the bacterial cells. (c) Actual movement, where the bacteria swim or move about in the suspending fluid. This last is what we mean by motility of a bacterium.

5. Make a hanging drop preparation from the broth culture of *Bacillus proteus vulgaris*.

(a) Ring about with vaseline, the depression on a hollow ground slide.

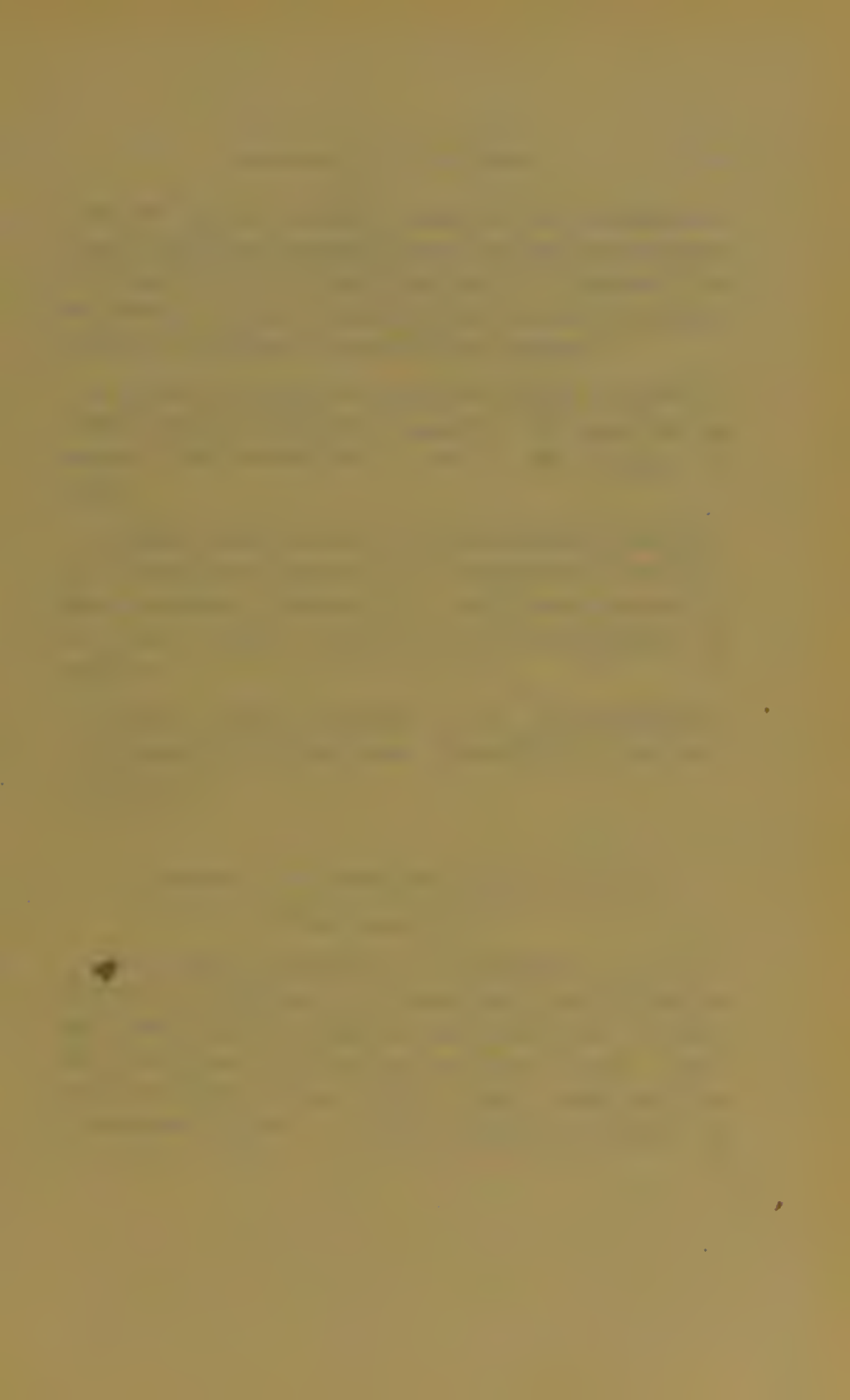
(b) Transfer a drop of the culture to a sterile cover glass, observing the usual precautions.

(c) Invert the cover glass over the cell and press firmly into the ring of vaseline so as to exclude the air.

In examining the preparation, find the edge of the drop with the low dry power, first almost closing the diaphragm, then fixing the slide, examine with the











high dry power, slightly opening the diaphragm. Usually sufficient magnification can thus be obtained for identity of form and motility. If desired, the preparation can be examined with the oil immersion lens, the diaphragm being further opened.

6. In like manner examine the broth culture of *Staphylococcus pyogenes aureus*. No motility is present, but Brownian movement can usually be noted.

7. Make potato cultures of the following moulds: (a) *Penicillium glaucum*, (b) *Aspergillus niger*, (c) *Mucor mucedo*. Keep at 21°C., and examine after 1, 2, 3, and 7 days, noting carefully characters of growth.

8. Make potato cultures of (a) *Saccharomyces cerevisiae*, (b) *Torula alba*. Keep at 21°C., and examine daily.

*Examination by dark-ground illumination.*

(Class demonstration).

The principle underlying the application of this method of examination is practically that by which dust particles are rendered visible in a ray of sunlight entering an otherwise darkened room. Bacteria and other minute objects when examined by this method appear as silvery particles against a

dark background, such features as size, shape and motility being readily made out. This method also renders visible certain bacteria not easily seen when unstained, or which are difficult to stain, e.g. certain of the spirochaetae.

Contrast the appearances by this method of examination, of preparations of bacteria previously examined.





## LESSON V.

Spores and spore staining—Examination of cultures of yeasts and moulds.

Agar or potato cultures of *Bacillus anthracis*, *Bacillus subtilis* and *Bacillus filamentosus* are supplied.

1. Make thin smears in usual manner from cultures of each of these bacteria. Stain for one to two minutes in Loeffler's methylene blue (Lesson II) and examine. The spores remain unstained as oval refracting bodies, either free or in the stained bacterial rods.

*Staining of spores.*

2. (a) Prepare thin smears of these bacteria in the usual manner, dry and fix in the flame.

(b) Place in Ziehl's carbol-fuchsin, or in anilin water fuchsin, for 20 to 30 minutes, keeping the staining fluid warm.

(c) Wash the films in water and then dip for two to three seconds in acid alcohol (Alcohol 97 cc., Hydrochloric acid 3 cc.), rinsing immediately after with water. The acid first decolorizes the rods, leaving the spores stained. At this stage the specimen may be examined by mounting in a drop of water and examining with high dry objective. If the bodies of the bacilli are still bright red, the film must be again

washed through the acid alcohol. If the spores are not stained, place the film again in the carbol-fuchsin.

(d) If the spores are properly stained, place the film for 15 seconds in Loeffler's methylene blue to stain the rods.

(c) Wash in water, dry thoroughly, and examine.

The spores are stained red, rods blue.

### *Examination of Yeasts and Moulds.*

7. With a stout platinum needle remove a small portion of the potato culture of *Penicillium glaucum*. Tease out in 5% caustic potash solution, or in a mixture of 4 parts alcohol and 1 of liquor ammonia, and examine under low and high dry objectives. From the alcohol ammonia mixture the specimens may be mounted and preserved in glycerine.

8. In similar manner examine the cultures of *Aspergillus niger* and *Mucor mucedo*.

Note the differences in fructification between these moulds.

9. Stain preparations of these moulds which have been teased out in the alcohol ammonia mixture, and then dried and fixed, using methylene blue. The mycelium and young spores stain, while the older spores do not.







10. Transfer with a platinum loop minute amounts of the culture of *Saccharomyces cerevisiae* to a drop of sterile water on a cover glass. Invert this on slide and examine with low and high dry, and the oil immersion objectives.

11. Repeat this procedure, using the culture of *Torula alba*.

Note the shape of the cells, the method of growth by budding, presence of vacuoles, etc.

12. Stain preparations of these yeasts prepared like bacteria (Lesson II), using Loeffler's methylene blue for one minute, as stain.

## LESSON VI.

Destruction of bacteria and spores by heat—Chemical disinfection—Cultures of *Bacillus anthracis*.

Broth cultures 48 hours old of *Staphylococcus pyogenes aureus* and *Bacillus proteus vulgaris*, also "spore threads" of *Bacillus anthracis* and *Bacillus subtilis* are supplied.

1. Place a double set of the cultures in the water bath at boiling temperature ( $100^{\circ}\text{C.}$ ), agitating the tubes so as to get even penetration of the heat. Remove one set at the end of 1 minute, the other at end of 2 minutes. Transfer 2 loops from each tube to sterile broth tubes and incubate the latter for two days at  $37^{\circ}\text{C.}$

2. Repeat this procedure but place the culture tubes in the water bath at  $80^{\circ}\text{C.}$  for 5 and 10 minutes respectively.

3. Repeat this procedure in the water bath held at  $60^{\circ}\text{C.}$  for 30 and 60 minutes.

4. Expose 3 sets of the spore threads in open dishes in the steam sterilizer, to the action of live steam for 10, 30 and 60 minutes respectively. On removal of each set drop a spore thread of each bacterium into separate tubes of sterile broth and incubate latter for two days.





5. Repeat above procedure except that the spore threads are placed in the hot air sterilizer at 100°C. for 10, 30 and 60 minutes. Contrast the findings in the broth cultures with those of preceding test.

6. Expose a set of the spore threads to a temperature of 110°C. in the autoclave for 20 minutes. On removal place in sterile broth and incubate.

### *Chemical disinfection.*

Sets of tubes are furnished containing respectively 10 cc. of 1% and 5% carbolic acid solution, and 1 in 1000 and 1 in 2500 solution of bichloride of mercury. Broth cultures of *Staphylococcus pyogenes aureus*, and a dilute suspension in sterile water of anthrax spores are also provided.

7. To each of one set of the tubes containing disinfectant solution, is added .5 cc. of the broth culture of *Staphylococcus pyogenes aureus* and the tubes are then well agitated. At the end of 5, 10, 20 and 60 minutes two loops are transferred from each tube to a tube of sterile broth. Incubate the broth tubes for three days, examining daily for evidence of growth.

8. In a similar manner add to each tube of a set of the disinfectant solutions .5 cc. of the suspension of anthrax spores, proceeding otherwise as in previous test.



## LESSON VII.

Bacillus anthracis in pure culture—Tissues of animal dead of anthrax septicaemia—Inoculation of animal with Bacillus anthracis.

1. Make smears from the agar, and the broth cultures of Bacillus anthracis. Stain one set with Loeffler's methylene blue, or diluted gentian violet, and others by Gram's method.

Spores if developed will be unstained, and appear as oval bodies free or in centre of rods.

2. Make a hanging drop preparation from the broth culture and examine for motility.

*Tissues of animal dead of anthrax septicaemia.*

Sections from lung, liver and kidney are supplied (in water).

3. Staining with Loeffler's methylene blue.

(a) Place sections in the stain for 5 to 10 minutes.

(b) Remove excess of stain by rinsing sections in water.

(c) Place in .5% acetic acid for 5 to 10 seconds, till the sections become a light blue.

(d) Immediately wash in fresh water.

(e) Dehydrate in absolute alcohol for 1 to 2 minutes.

(f) Clear in xylol for 1 to 2 minutes.

(g) Transfer to slide with section lifter and mount in Canada balsam.

Sections after dehydration may be transferred to slide and cleared there with xylol before mounting, as xylol causes at times considerable shrinkage and curling.

Examine with the low and high dry powers and the oil immersion objective. Anthrax bacilli are seen in the capillaries of the organs.

#### 4. Staining by Gram's method and eosin.

(a) Place the sections in alcohol for 1 minute.

(b) Transfer to anilin gentian violet, 5 to 10 minutes.

(c) Rinse the section in distilled water, and then place for 5 minutes in Gram's iodine solution.

(d) Rinse in water, and then in alcohol for  $\frac{1}{2}$  minute.

(e) Place for 1 minute in eosin staining fluid.

Alcoholic eosin ..... .5 gramme.

Alcohol . . . . . 70 cc.

Water . . . . . 30 cc.







(f) Remove excess of eosin by washing in water and then in alcohol.

(g) Rinse in acid alcohol for 2 seconds, and immediately transfer to alcohol till the sections become red.

(h) Clear in xylol, transfer to slide and mount in Canada balsam.

*Inoculation of mice or guinea pigs subcutaneously.*

(Class demonstration).

The animal is placed in a holder or held by an assistant. The hair is clipped from the point of inoculation (root of tail in mouse, inner side of thigh in guinea pig) and the skin at this point is washed with alcohol. With a sterile scissors a small snip is made through the skin. With a platinum needle a small loop of 48 hours agar culture of *Bacillus anthracis* is introduced and pushed well up under the skin. The point of introduction is then lightly seared with a heated glass rod. Mice usually die within 24 hours; guinea pigs within 48 hours.

## LESSON VIII.

Examination of guinea pig dead of anthrax septicaemia—  
Plate cultures—Cultures of the pyogenic bacteria.

*Guinea pig dead of anthrax.*

1. (a) Carefully examine the animal, noting the swelling extending from the groin, to a variable extent over the abdomen. Then stretch out the animal by tacking down the legs to a board moistened with 1 in 1000 bichloride of mercury solution.

(b) Lay the hair of the animal by moistening with the bichloride solution.

(c) With a sterilized scissors carefully cut through the skin along the median line and reflect it from over the thorax, abdomen and thighs.

(d) Note the situation and character of the oedema. From the oedema fluid, make smears for after staining. (Cultures might also be made).

(e) Sterilize the abdominal wall in the midline and the thorax at the rib-costal junctions, with a flat-bladed knife, heated to redness.

(f) With sterilized forceps and scissors open into the abdomen along the midline. With fresh forceps remove the spleen and break a portion of it up in a sterile capsule. Place a drop of pulp on slide and with a second slide spread out the drop into a thin film, and then carefully draw the slides apart.





(g) Open carefully the thorax, cutting through the costal cartilages, and expose the heart.

(h) Sterilize a portion of the exposed heart wall by searing it with a heated glass rod. Push through this part a sterile pipette and withdraw the blood of the chambers. Several pipettes may be filled in this manner, from one of which smear preparations can be made as described in (f) for staining. On withdrawing pipettes fuse the open ends carefully in the flame. Preserve the pipettes for the making of plate cultures.

The remaining viscera of the animal may be removed and preserved in 80% alcohol for the after study of sections.

*Staining of the tissue and blood smears.*

Fix the films after drying by passing through the flame. Better results are secured in staining the preparations from the spleen pulp and the blood, by fixing the films in equal parts of ether and absolute alcohol, or in absolute methyl alcohol. Heating the films for 20 to 30 minutes to 110°C. in a hot air oven secures good fixation and is preferred by many.

2. Staining with eosin and methylene blue.

(a) Place the fixed films in eosin  $1\frac{1}{2}$  to 1 minute.

(b) Wash in water and after removing excess of water place in Loeffler's methylene blue for 1 to 2 minutes.

(c) Wash in water, dry and examine with the dry and oil immersion objectives.

Anthrax bacilli and nuclei are stained blue, the red corpuscles and eosinophile granules of leucocytes, pink. Spores are not seen in the animal body.

3. Staining by Gram's method and eosin,

(a) Place the fixed films in anilin gentian violet for 3 minutes.

(b) Rinse in water and transfer to Gram's iodine for 3 minutes.

(c) Rinse again in water and then in alcohol till stain ceases to come away.

(d) Place in eosin,  $\frac{1}{2}$  to 1 minute.

(e) Wash in water, dry and examine.

#### *Plate Cultures.*

4. Make plate cultivations from blood of heart of guinea pig dead of anthrax septicaemia.

(a) Liquefy tubes of agar by boiling in water bath. Cool down to 42 to 45°C.

(b) Observing all aseptic precautions with a sterilized forceps break off the fused end of pipette containing blood from the heart, and allow one or more drops of blood (according to number of bacteria found present by microscopic examination), to fall into a tube of sterile water or salt solution.







(c) From this tube after careful admixture of the blood, transfer three loops to a tube of the liquefied agar.

(d) Heat the top of the inoculated tube to assure thorough sterilization of lip of tube.

(e) Remove the plug and carefully pour the agar into a sterilized petri dish, causing it to spread thoroughly over the plate surface.

(f) Allow the agar to set thoroughly and then invert the plate and place in the incubator at 37°C.

Examine at the end of 24, 48 and 72 hours, noting the character and number of colonies, and the presence or absence of other species of bacteria.

Esmarch roll tubes may be prepared instead of plates, by spreading the infected liquefied agar over the sides of the tubes by rapidly revolving the tubes on ice.

#### *Cultures of the pyogenic micrococci.*

5. Make cultures of *Staphylococcus pyogenes aureus*, *Staphylococcus pyogenes albus* and *Bacillus pyocyaneus* in agar, potato and gelatine (stab) tubes.

6. Make cultures of *Streptococcus pyogenes* in agar, and broth tubes.

Incubate all except the gelatine tubes at 37°C.

Examine the cultures day by day, noting the general characters of growth.

## LESSON IX.

Plates from anthrax blood—Pyogenic micrococci—Staining of pus—Pus plates—Intravenous inoculation.

1. Examine the anthrax plate cultures and note size, shape and outline of colonies which appear. Make smears from a number of colonies and study morphology of bacteria. Subculture on agar and broth two colonies with the morphology of the anthrax bacillus.

2. Prepare smears from the agar or potato cultures of *Staphylococcus pyogenes aureus*, *albus*, and *Bacillus pyocyaneus*.

Stain with methylene blue or anilin gentian violet, and also Gram's method.

3. Prepare smears from the agar and the broth cultures of *Streptococcus pyogenes*.

Stain with methylene blue and by Gram's method. Contrast the appearances microscopically.

*Staining of Pus from an Abscess.*

4. Make smears of pus from an abscess in a manner similar to that employed for splenic pulp and blood of animal dead of anthrax septicaemia (Lesson VIII). Dry and fix in usual manner.

Stain these films by following methods:





(a) with eosin and methylene blue (page 29) ;

(b) by Gram's method and eosin (page 30) ;

(c) with methylene blue alone, treating as bacterial smear (Lesson II). Contrast the appearances and findings by these methods.

*Plate cultures from pus.*

5. Inoculate a few cc. of sterile water or broth with one or more loops of the pus furnished, according to number of bacteria found by microscopic examination. Proceed in other respects as in making plates from anthrax blood.

Incubate the plates at 37°C., examining after 24 to 48 hours.

N.B.—The use of plates in ordinary practice is not necessary, as tubes can be smeared either directly from the pus, using minute amounts, or, better, can be smeared from the dilution in sterile water or broth.

*Intravenous and subcutaneous inoculation.*

(Class demonstration).

6. After usual preparation of site, inoculate into the posterior auricular vein of a rabbit's ear .25 cc. of a 48 hour broth culture of *Staphylococcus pyogenes aureus*.

7. After usual preparation, carefully inoculate .1 cc. of 48 hour broth culture of freshly isolated *Streptococcus pyogenes* under skin of ear of rabbit.

Examine these animals carefully morning and evening. The former animal usually dies after 48 hours, and should be carefully examined post-mortem, and bacteriological examination made of lesions as in anthrax.







## LESSON X.

Examination of plate cultures from pus—Effects of disinfection on bacteria of hands—Pneumococcus—

Pneumonic sputum.

1. Examine the plate cultures from pus, made last day, making smear preparations from any different varieties of bacteria developing on them. Stain with methylene blue and also by Gram's method.

2. Make agar and broth sub-cultures of any different varieties of bacteria that may be present. Examine the cultures daily, noting characters of growth so as to aid identity of the species.

*Effects of disinfection on bacteria of hands.*

3. With a sterilized knife scrape some of the epidermal scales from front of fingers or from under a finger-nail, into a tube of sterile broth. After thorough admixture transfer 3 loops to a tube of liquefied agar and make a plate culture. (See page 30).

4. Repeat this procedure on hand disinfected by any recognized surgical method. Add however .5 cc. of the broth dilution instead of 3 loops. The broth tube should also be incubated.

*Pneumococcus and pneumonic sputum.*

5. Make cultures on blood agar and inulin serum-water tubes from the cultures of *Pneumococcus* and *Streptococcus pyogenes* furnished.

Incubate at 37°C. for 3 days, noting daily the difference in cultural characters.

6. Make smears from the blood serum and the serum-water cultures of the *Pneumococcus* furnished, staining by Loeffler's methylene blue and by Gram's method. Contrast the appearances microscopically with preparations of *Streptococcus pyogenes*.

7. Prepare smears from the pneumonic sputum in the same manner as pus smears were made, (fresh sputum should be obtained if possible, but if not available, sputum preserved in 80% alcohol may be used). Owing to tenacious character of the sputum, forceps are advisable to pick up portions selected for examination. Stain the smears so prepared, by Gram's method, counterstaining in eosin (Page 30).

This method stains the capsule fairly well.

8. Stain other prepared films of the pneumonic sputum, in eosin and methylene blue (Page 29).

9. To bring out the capsule in fresh sputum, use the following method:





(a) Prepare films in the usual manner, dry and fix in the flame.

(b) Pass the films quickly through glacial acetic acid.

(c) Remove excess of acid and, without washing place the film in anilin gentian violet for 5 to 8 minutes, agitating the smear in stain so as to wash off any remnant of the acid.

(d) Rinse in water, dry and examine.

The capsule shows up better if film is mounted in water.

## LESSON XI.

Plate cultures from hands—Gonococcus and gonorrhœal pus  
—Pus from actinomycosis—Cultures of Streptothrix  
actinomyces.

1. Examine the plates made last lesson from disinfected and non-disinfected hand. Contrast the numbers and characters of the colonies. Make smears and stain by Loeffler's methylene blue and by Gram's method.

2. Make agar and broth sub-cultures of any different varieties of bacteria on the plates, noting characters of growth and attempt to identify the species.

*Gonococcus and Gonorrhœal Pus.*

In the usual manner prepare smears of the pus furnished. It is best to make smears directly from the discharge.

3. Stain the dried and fixed smears in eosin and methylene blue (page 29).

Note the relationship of the gonococci to the pus cells.

4. Stain other smears by Gram's method (page 13), counterstaining for 1 minute in Bismarck brown (1% aqueous solution) before final drying.

Gonococci are stained brown, other pyogenic micrococci violet.







5. Make smears from the blood agar cultures of the gonococcus furnished. Stain with Loeffler's methylene blue. Contrast the morphology of the bacteria in this culture, with those in the pus.

*Pus from actinomycosis.*

Note the minute greyish yellow granules in the pus.

6. Place on a slide a drop of the pus, containing one or more granules. Treat it with several drops 5% caustic potash solution and press on a cover glass.

Examine with low and high dry objectives. Radiating masses of the streptothrix and separate threads can readily be detected.

In pus from bovine actinomycosis distinct clubs at the periphery of the masses are usually found. These are only occasionally seen in pus from lesions in human subject.

7. Prepare smears from the pus, and stain,

(a) By Gram's method and eosin (page 30), increasing time in stain and in iodine to 10 minutes.

(b) By Weigert's modification of Gram's method.

(a) Place the films in alcohol 1 to 2 minutes.

(b) Transfer to anilin gentian violet, 10 to 15 minutes.

(c) Rinse in water and then transfer for 10 minutes to Weigert's iodine solution (Iodine 1, Potassium iodide 2, Water 100).

(d) Rinse again in water. Counterstain in eosin for 1 minute.

(e) Wash in water and remove water with blotting paper.

(f) Dehydrate and decolorize in a solution of anilin oil 2 parts, xylol 1 part, till violet color has almost disappeared.

(g) Wash in xylol to remove anilin oil and then mount in Canada balsam.

Examine with low and high dry objectives and with the oil-immersion lens. The threads will be stained purple, clubs if present red.

9. From the agar cultures of *Streptothrix actinomyces* furnished, make smears and stain by Gram's method (page 13), doubling time in the stain.

Contrast the morphology of the streptothrix in culture with that in the pus.





## LESSON XII.

Meningococcus—Influenza bacillus in sputum—Micrococcus catarrhalis—Tissues containing pyogenic micrococci—  
Cultures from normal throats—Cultures of  
Bacillus diphtheriae.

1. Make smears from the blood serum cultures of the meningococcus (*Micrococcus intracellularis*) furnished. Stain by Loeffler's methylene blue and by Gram's method.

If obtainable, smears should also be examined from the cerebro-spinal fluid secured by lumbar puncture from a case of epidemic meningitis, or from the meningeal pus of a fatal case. These smears should be stained similar to, and contrasted with, those of gonorrhoeal pus (page 38).

2. From the sputum of a case of influenza bronchitis prepare smears in usual manner. Stain in methylene blue alone, or by eosin and methylene blue (page 29), and also by Gram's method and eosin.

The influenza bacilli appear as fine short rods, at times fine segmented threads.

3. Make smears from the agar culture of *Micrococcus catarrhalis* furnished. Stain by Loeffler's methylene blue and by Gram's method. Contrast with the preparations of the gonococcus and meningococcus.

*Staining of tissues for pyogenic micrococci.*

4. Stain sections of erysipelas of rabbit's ear.

(a) By Gram's method, counterstaining in eosin (page 26).

(b) With Loeffler's methylene blue (page 25).

5. Stain the sections of liver and kidney from rabbit killed by intravenous inoculation of *Staphylococcus pyogenes aureus*.

(a) With methylene blue (page 25).

(b) By Gram's method counterstaining in eosin (page 26).

These methods may be employed for other tissues, containing the pyogenic micrococci or the pneumococcus.

*Cultures from normal throats.*

6. Rub a sterile swab over the tonsillar surface, giving swab a rotary motion. The swab should then be lightly rubbed over a blood serum tube which is incubated for 24 hours at 37°C. The same or a second swab is then used to make smears on slides, one being stained by Loeffler's methylene blue, another by Gram's method and eosin (page 30). Carefully note the bacterial forms present and contrast with bacteria which develop on culture tube.







7. From the culture of *Bacillus diphtheriae* furnished, inoculate

- (a) a blood serum tube,
- (b) a glycerine agar tube,
- (c) a broth tube.

Incubate at 37°C., examining at end of 18, 24 and 48 hours.

## LESSON XIII.

*Bacillus diphtheriae*—Diphtheroid bacilli—Diphtheria membrane—Inoculation of animals with diphtheria bacilli—  
—Diphtheria toxin and antitoxin.

1. Make smears from the cultures of *Bacillus diphtheriae* on the blood serum, glycerine agar and broth tubes.

Stain with Loeffler's methylene blue, and also by Gram's method. Note the variable morphology of the bacilli, and the variations in this respect between the growths on different media.

2. Stain films from 18 to 24 hour blood serum cultures by Neisser's method.

(a) Place the fixed films for  $\frac{1}{2}$  to 1 minute in following stain:

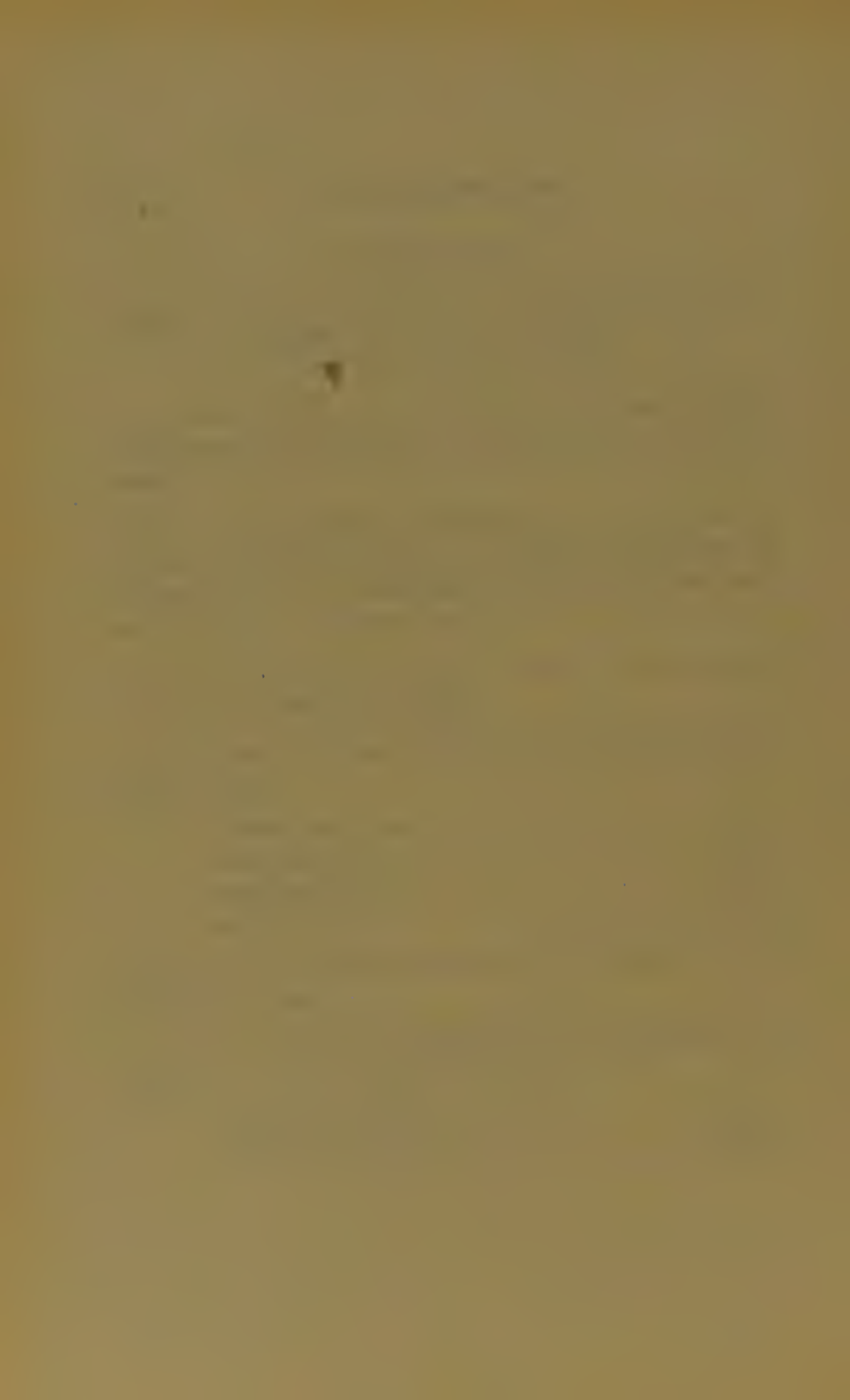
Glacial acetic acid . . . . .	5 cc.
Distilled water . . . . .	95 cc.
Methylene blue . . . . .	.1 gm.
Alcohol 96% . . . . .	2 cc.

Dissolve the methylene blue in the alcohol and add the acid water.

(b) Rinse quickly through water and place for  $\frac{1}{2}$  minute in stain made as follows:

Bismarck brown . . . . .	1 gm.
Boiling distilled water . . . . .	500 cc.





(c) Rinse again in water, dry and mount in balsam. The bacilli are brown with one or two blue bodies usually at ends. Pseudo-diphtheria bacilli usually exhibit no such bodies.

This stain has no special advantage over Loeffler's blue.

3. Make smears from the blood serum culture of *Bacillus xerosis* (or other diphtheroid bacillus) furnished. Stain by Loeffler's methylene blue, by Gram's method and by Neisser's method, and contrast with similar preparations of the diphtheria bacillus.

4. Make smears from the blood serum cultures furnished, from clinical cases of diphtheria. Stain with Loeffler's methylene blue. Note presence or absence of diphtheria bacilli, and any other forms of bacteria present.

### *Diphtheria Membrane.*

5. Stain the sections of diphtheria membrane furnished, (in water).

(a) By Gram's method counterstaining in eosin (page 26).

(b) By eosin and methylene blue.

(1) Place the sections in a 5% aqueous solution of eosin for 20 minutes.

(2) Wash in water and place in Loeffler's methylene blue for 5 minutes.

(3) Wash again in water and then dehydrate by ordinary and absolute alcohols, clear in xylol, and mount in usual manner.

The diphtheria bacilli (usually accompanied by other bacteria) are found in the superficial layer of the membrane.

*Inoculation of diphtheria bacilli.*

(Class demonstration).

6. A medium sized guinea pig (250 grammes) is inoculated subcutaneously with .5 cc. of a 48 hour broth culture of *Bacillus diphtheriae*. The animal usually dies inside 48 hours.

Make careful notes of the conditions found at autopsy, and make bacterial examination of tissues at point of injection, of blood and spleen as in anthrax. (Page 28).

7. Two 250 gramme guinea pigs are inoculated subcutaneously with 1 cc. each of 48 hour broth culture of the *Bacillus diphtheriae* and at the same time are given, one about 50 and the other 100 units of diphtheria antitoxin. (This amount of antitoxin can be approximately secured by measuring a vial or tube of antitoxin and calculating from its labelled strength in units the amount requisite).







As a rule both recover, though occasionally the animal treated with the smaller dose dies, if the diphtheria bacillus has manufactured a virulent toxin.

8. Inject a 250 gramme guinea pig with .5 cc. of a 48 hour broth culture of *Bacillus xerosis*. The animal suffers no ill-effects.

## LESSON XIV.

Pure cultures of the tubercle bacillus—Tubercular sputum.

1. From the blood serum cultures of the tubercle bacillus furnished, prepare smears and stain in the following manner:

(a) Place in Ziehl's carbol fuchsin for 5 minutes.

(b) Wash in water and then rinse quickly through 20% nitric acid solution.

(c) Wash immediately in water and then for 1 minute in 70% alcohol.

(d) Wash in water, dry and examine.

2. Prepare smears from the blood serum cultures of bovine tubercle bacillus furnished. Stain by same method as above.

The bovine bacilli are as a rule somewhat shorter and straighter than the human variety.

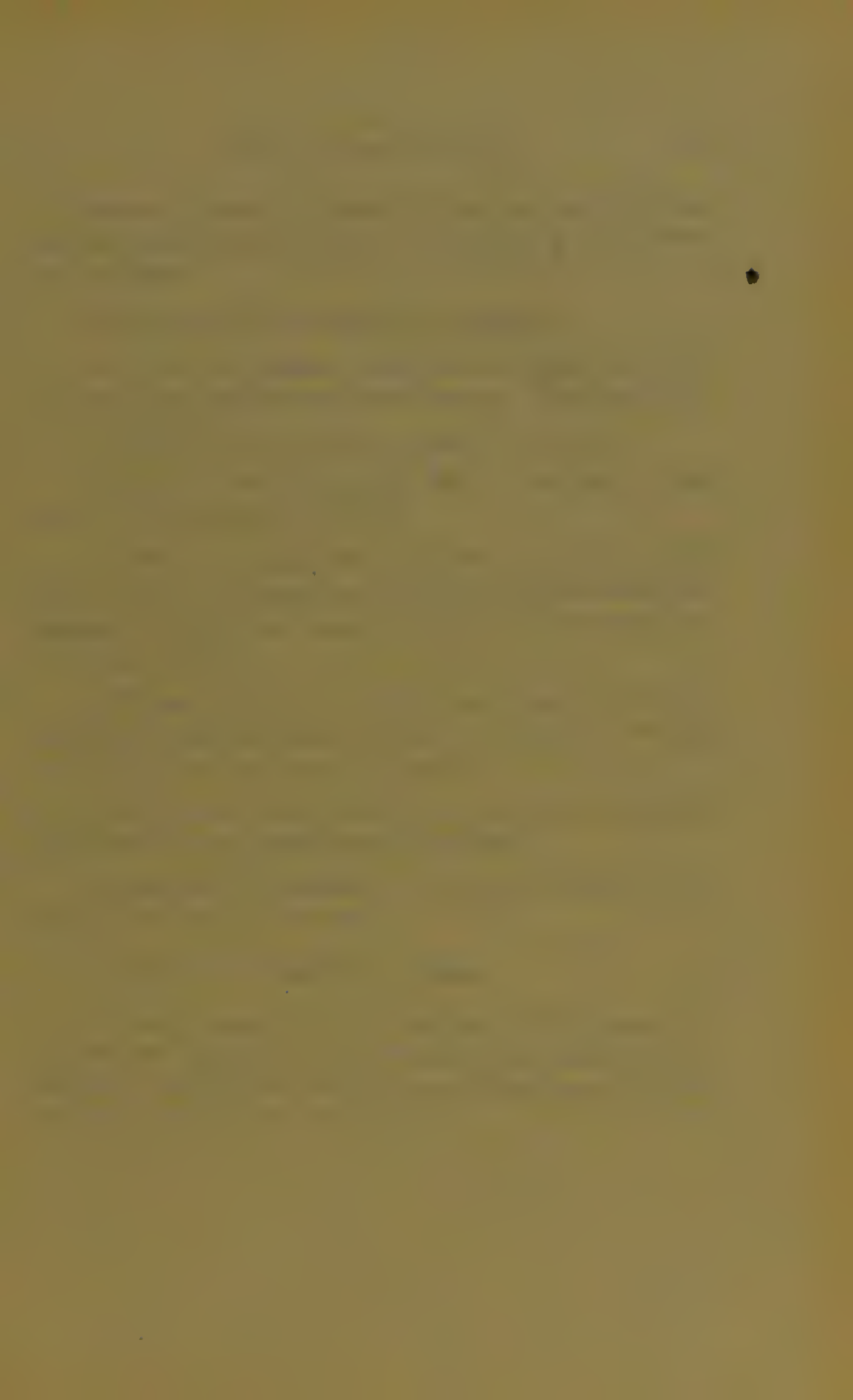
3. Prepare other smears and stain by Gram's method (page 13), leaving films in stain four times the usual period.

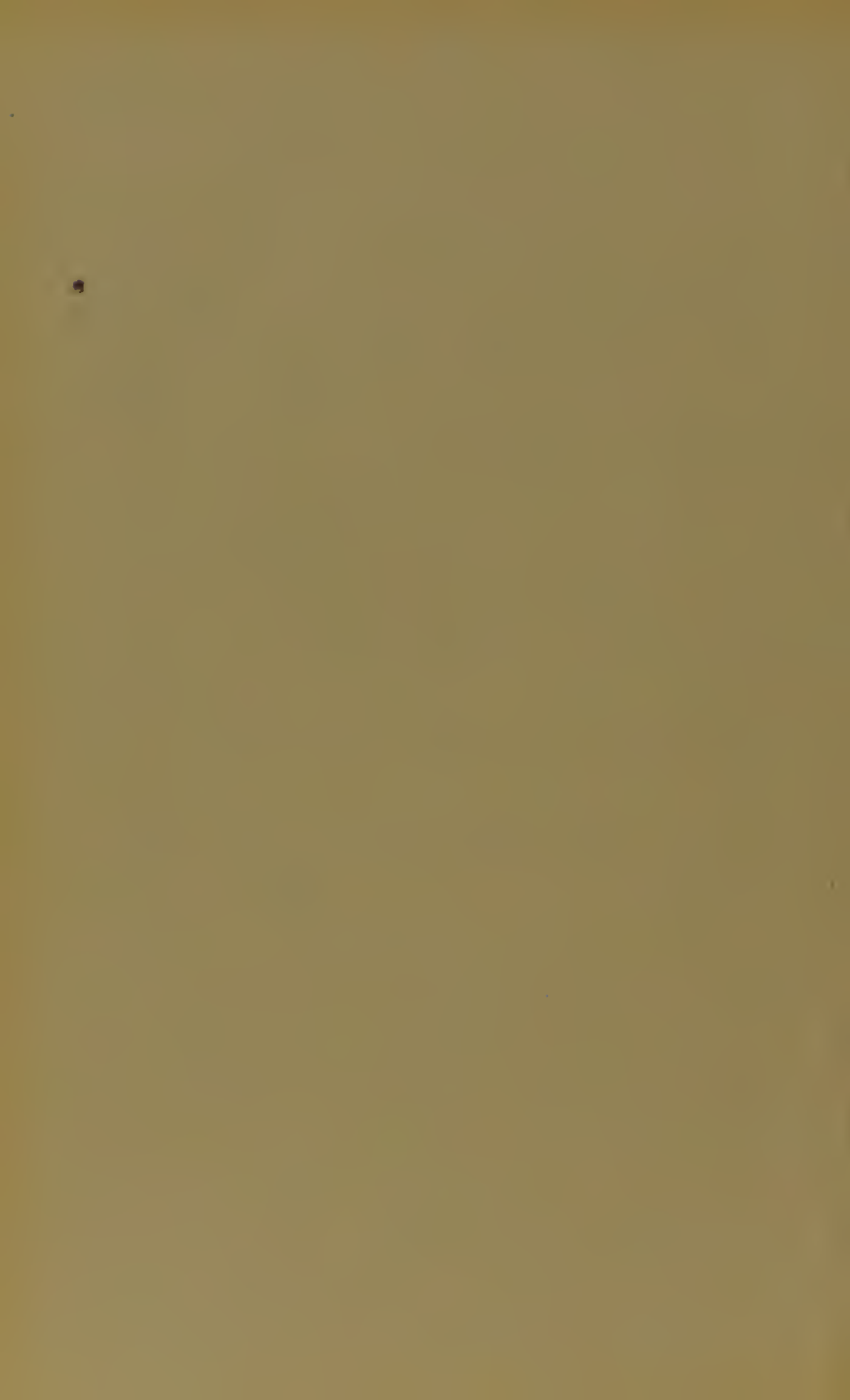
*Examination of sputum for tubercle bacilli.*

Pour a thin layer of sputum into a glass capsule and pick out for examination a yellowish (caseous) particle if present; otherwise choose a purulent part. Forceps will be found best for picking up particles desired.











Prepare smears by pressing out the particles between two slides as for pus, then drying and fixing in the flame.

#### 4. Staining by Ziehl-Neelsen's method.

(a) Cover the smears with carbol-fuchsin for 5 to 10 minutes, keeping the stain warm. (Do not boil).

(b) Wash off the excess of stain in water.

(c) Rinse quickly through 20% nitric acid. The red color disappears at once.

(d) Wash immediately in water and then in 70% alcohol for 1 to 2 minutes. If the red color which reappears is persistent, again pass the film through the acid and alcohol.

(e) Wash the film in water and counterstain in Loeffler's methylene blue for  $\frac{1}{4}$  to  $\frac{1}{2}$  minute. Wash in water, dry and mount as usual.

Tubercle bacilli appear as red rods; other bacteria and the nuclei of cells are stained blue.

This method is decidedly the most accurate for routine examination work.

#### 5. Staining by Gabbett's method.

The same method of procedure is followed till after the removal of the smear from the carbol-fuchsin. It is rinsed in water and then placed for 30 seconds in Gabbett's solution:

Methylene blue . . . . .	2 grammes.
Sulphuric acid . . . . .	25 cc.
Water . . . . .	75 cc.

The smear is then washed in water, dried and mounted in the usual manner.

This method is shorter but it has not proven so accurate, nor does it give as clear a film as the Ziehl-Neelsen process.

#### 6. Staining by Herman's method.

(a) Cover the smears for 5 to 10 minutes with Herman's stain and keep warm. This stain is a 3% solution of crystal violet in 95% alcohol; to one part of this is added, at time of use, three parts of 1% aqueous solution of ammonium carbonate.

(b) Wash in water and place in 10% nitric acid for a few seconds.

(c) Immediately rinse in water and then in 95% alcohol for 1 to 2 minutes. All violet color should be gone but if not rinse again through the acid and alcohol.

(d) Counterstain for 10 seconds in .3% aqueous solution of chrysoidin. Wash and dry.

The tubercle bacillus appear as violet bacilli on a yellow ground. This method gives results about equal to the Ziehl-Neelsen process, but is not quite so easy of application.





*Examination of sputum or fluids containing few or no tubercle bacilli.*

When tubercle bacilli are suspected but cannot be found by examination of material chosen directly, the following method is advisable for concentration:

Equal parts of sputum and 30% solution of anti-formin are thoroughly mixed and allowed to stand from one to twelve hours to digest. A more rapid process is to warm the mixture to about 50°C. and shake for 5 to 10 minutes in the sputum shaking machine. The mixture in either case is then centrifuged, the liquid portion decanted and the sediment washed with sterile, freshly distilled water so as to remove the remnants of antiformin. The centrifuged sediment is then used to make smears which are stained by methods already described.

The antiformin method can also be used on pus or faeces, making the strength of solution from 15 to 20%. It is also applicable to tissues, such being ground up in the antiformin to aid solution.

In urine, smears are made from the centrifuged sediment, without preliminary treatment with antiformin, (unless cultures are to be made). As smegma bacilli give staining reactions similar to the tubercle bacillus, it is advisable to examine specimens drawn by catheter. Further, in staining these smears by Ziehl-Neelsen process, it is advisable to use full strength alcohol in place of 70%, as this de-

colorizes the smegma bacillus but does not affect the tubercle bacillus. Smears from urine sediment should always be cleared after fixation by placing in warm water at 65° to 70°C. for 5 minutes; ammoniacal urines with phosphate deposits should in addition be cleared in 5% acetic acid.

Antiformin kills practically all bacteria of the sputum inside 30 to 40 minutes, except the tubercle bacillus, which is protected by its waxy envelope and will withstand its destructive action well over one hour. Hence the washed centrifuged deposit may be used to smear tubes of Dorset's egg media or blood serum, and so obtain cultures from sputum.

When microscopic methods fail, inoculation of a guinea pig subcutaneously with the sediment, will lead to the development of tuberculosis, if any living bacilli are inoculated.







## LESSON XV.

Acid-fast bacteria—*Bacillus leprae* (Duval)—Tubercular tissues—Leprosy tissues—Cultures of acid-fast bacteria—Cultures of *Spirilla*.

1. From the agar culture of Moeller's grass bacillus furnished, make smears and stain as for tubercle bacillus (page 48).

2. Make smears from the smegma of guinea pigs and stain as for tubercle bacillus.

As a rule numbers of acid fast bacilli are present.

3. From the blood serum culture of *Bacillus leprae* (Duval) furnished, make smears and stain as for tubercle bacillus.

Contrast the appearances presented by above bacteria with preparations of the tubercle bacillus.

*Tubercle bacilli in tissues.*

Sections are furnished (in water), from acute tuberculosis of lung, and also of tuberculosis of lung of cattle.

4. Staining in fuchsin solutions.

(a) Place the sections in carbol-fuchsin or in anilin fuchsin for from 30 minutes to 24 hours, keeping the stain covered in the incubator at 37°C. Better results are obtained by leaving in stain for 24 hours.

(b) Rinse the sections in water, and then transfer for a few seconds to 20% nitric acid.

(c) Wash at once in water and then in 70% alcohol till no more red stain comes away. If the section is still bright red, wash again through the acid and alcohol.

(d) Rinse in water and place in Loeffler's methylene blue for 1 minute.

(e) Rinse again in water, dehydrate in absolute alcohol, clear in xylol and mount in usual manner.

Sections from paraffin blocks, received into water at 48°C., may be fixed on cover glasses and stained as cover glass films in the following manner:

Float a section on a cover glass and, removing from water, blot firmly and evenly with several layers of blotting paper. Heat the section carefully to melt the paraffin, rinse through xylol to free the section from the paraffin, then in alcohol to free from xylol. From the alcohol place directly into the carbol-fuchsin and proceed as above.

### *Leprosy Tissues.*

*Bacillus leprae* stains like the tubercle bacillus, but more readily and is more rapidly decolorized.

5. Stain sections by the same method as tubercular tissues.





The leprosy bacilli are always present in large numbers in the leprous nodules, and the discharges in ulcerating tubercular leprosy.

*Cultures of acid-fast bacteria.*

6. From the tubes of human and bovine tubercle bacilli furnished, make cultures on

- (a) Dorset's egg media;
- (b) Dorset's glycerine egg media.

After inoculation cover the tubes with rubber caps soaked in bichloride solution. Incubate at 37°C. and examine at end of 7, 10, 14 and 21 days.

7. From the tubes of Moellers grass bacillus and *Bacillus leprae* (Duval) furnished, make cultures on

- (a) Dorset's egg media;
- (b) agar.

Incubate at 37°C. and examine daily.

*Cultures of spirilla.*

8. Make inoculation of *Spirillum cholerae asiaticae*, *Spirillum metschnikowi*, and *Spirillum* of Finkler and Prior on,

- (a) agar tubes (slope);
- (b) peptone tubes;
- (c) gelatine tubes (stab).

Incubate two former at 37°C., latter at 21°C.

## LESSON XVI.

*Spirillum* of asiatic cholera—*Spirillum* of Finkler and Prior—*Spirillum metschnikowi*—Indol test—Agglutination tests—Cultures of *Bacillus typhosus* and *Bacillus coli*.

1. Examine hanging drop preparations from the peptone cultures, of each of the spirilla.

2. Make smears from the agar and the peptone cultures, of each of the spirilla. Stain with anilin gentian violet for 30 seconds and mount in the usual manner. Contrast the preparations from different culture media.

3. To the peptone cultures of each of the spirilla add carefully, with a pipette, 10 drops of sulphuric acid.

A rose red coloration appears with *Spirillum cholerae* and *Spirillum metschnikowi*, but not with the *Spirillum* of Finkler and Prior. This is due to the formation by the two former of indol and nitrites. As indol only gives this reaction in the presence of nitrites, if no reaction follows the addition of the acid, add 1 cc. of a .01% solution of potassium nitrite, freshly prepared. If the reaction does not then develop, indol is certainly absent.

4. On a sterile cover glass place a loop of the peptone culture of *Spirillum cholerae asiaticae*. This culture should not be more than 18 hours old and







free from clumps. Add a loop of 1 in 50 dilution of serum of rabbit immunized against the cholera spirillum. Examine in hanging drop using high dry lens. Loss of motility and agglutination of the spirilla occurs in from 1 to 15 minutes.

5. Repeat, substituting loops of the other spirilla for the cholera spirillum. No agglutination occurs.

6. From the tubes furnished make cultures of *Bacillus typhosus* and *Bacillus coli* on,

- (a) agar;
- (b) potato;
- (c) litmus milk;
- (d) broth or peptone;
- (e) dextrose broth fermentation tubes;
- (f) lactose neutral red broth fermentation tubes;
- (g) lactose bile fermentation tubes.

Incubate at 37°C. Contrast the growths from day to day.

## LESSON XVII.

Cultures of *Bacillus typhosus* and *Bacillus coli*—Typhoid tissues—Examination of faeces.

1. Compare hanging drop preparations from the broth cultures of *Bacillus typhosus* and *Bacillus coli communis*.

2. Examine the three-day old pèptone cultures of *Bacillus typhosus* and *Bacillus coli communis* for indol, adding to each tube 1 cc. of a .01% fresh solution of potassium nitrite and 10 drops of sulphuric acid.

*Bacillus coli* gives the indol reaction.

3. Make smears of *Bacillus typhosus* and *Bacillus coli* from the agar, potato and broth cultures. Stain in anilin gentian violet or in Loeffler's methylene blue, and contrast the appearances from different media. Note also the reaction of these bacilli to Gram's stain.

*Typhoid Tissues.*

4. Sections of mesenteric gland, spleen and liver from typhoid fever cadaver are furnished, (in water).

In the tissues *Bacillus typhosus* is found scattered in clumps, so that a number of sections may have to be examined before finding any bacilli. At least six sections of each of the tissues should be stained.





Stain in Loeffler's methylene blue, leaving in the stain 20 to 30 minutes. Rinse in water and then in .1% acetic acid for  $\frac{1}{4}$  minute; again rinse in water to remove acid, dehydrate in absolute alcohol, clear in xylol and mount in balsam.

*Examination of Faeces.*

An emulsion of human faeces (1 part in 10 parts water) is furnished.

5. Make smears from this emulsion. Stain by Loeffler's methylene blue, by Gram's method and in Weigert's iodine solution. Contrast the findings, noting forms and numbers of bacteria present.

6. Transfer one loop of this emulsion and one loop of a typhoid broth culture to a tube containing 10 cc. of sterile water. After thorough admixture add one loop to tubes of liquefied agar, and agar containing 1 to 100,000 gentian violet and make plates (page 30).

Incubate at 37°C. and note any differences in development.

7. With a bent glass rod dipped in the dilution of faecal emulsion lightly rub the surface of the plate containing Drigalski and Conradi's medium. Incubate at 37°C. and note character of colonies which develop.

8. Add .1 cc. of the dilution of faeces to fermentation tubes of glucose broth, neutral red lactose broth and lactose bile. Incubate at 37°C. for 2 days, noting appearances daily.

## LESSON XVIII.

Examination of faecal plates—Serum diagnosis of typhoid fever or the Widal reaction—Cultures of *Bacillus dysenteriae*, *Bacillus paratyphosus* and *Bacillus enteriditis*.

1. Examine the various plate cultures from faeces and stain smears from any different varieties of colonies present.

2. Study in hanging drop, the motility of the bacteria in a number of colonies which resemble the typhoid or colon bacillus. This is done by transferring a minute portion from a colony to a drop of broth or sterile salt solution on a cover glass, and making a hanging drop.

3. From the Drigalski plate, examine as above at least three "blue" colonies. If morphology corresponds to the typhoid bacillus, transfer a loop from a colony to tube containing 2 cc. of sterile salt solution and thoroughly mix. Test this bacillus against a known typhoid bacillus with typhoid blood serum as described below, or with a typhoid serum from an immunized animal as for the cholera spirillum (page 56).

*The serum diagnosis of typhoid fever or the Widal reaction.*

In the blood serum of animals immunized against *Bacillus typhosus*, and also, at an earlier or later







date, in the blood of persons with typhoid fever, there are present certain constituents which possess the specific property of agglutinating typhoid bacilli. The phenomena noted microscopically are loss of motility of the bacilli and their aggregation in clumps. In tube experiments, we have precipitation of the contained bacilli.

Cultures employed are usually 18 to 24 hour broth cultures or a suspension in normal salt solution, from a fresh agar culture. The serum may be obtained by collecting blood in a Wright pipette, or in small wide-mouthed vials, and allowing blood to clot and serum to separate. The centrifuge may be used to assist separation. Commonly, however, medium-sized drops of blood are allowed to dry on slides and the serum is later extracted by breaking up blood drop in sterile water, salt solution or broth. As it is necessary to dilute the serum at least 30 times in making the test for diagnosis, more accurate dilution can be made from the serum itself. But in Laboratories of Boards of Health, the dried blood method is usually employed as more universally applicable and sufficiently accurate for clinical purposes.

Medium sized blood drops dried on slides and obtained from the lobe of the ear or finger of a case of typhoid fever, are furnished.

4. (a) With a platinum loop or a capillary pipette add carefully 8 drops of sterile water (or broth) to a

dried blood drop, and carefully break up blood drop so as to extract the agglutinating constituents. The drops added should equal the blood drop in size. This gives a dilution of about 1 in 16. The mixture should be of a light straw color.

(b) On a sterile cover glass, place one loop of this extract, and add one loop of the 18 hour broth culture of *Bacillus typhosus* furnished. Make a hanging drop preparation and place in the incubator at 37°C. Examine with high dry lens after 30 minutes and at end of hour. Agglutination ought to be marked by latter period if blood contains the specific agglutinins.

5. Repeat this procedure, using one loop of the extract, one loop of sterile broth and one of the typhoid culture, (giving a dilution of about 1 in 60).

Examine as before and note results.

Further dilutions can be made and results studied.

6. Repeat this procedure, using one loop of the extract and one of the suspension in salt solution of a blue colony from the Drigalski plate. Note if agglutination occurs.

The reaction can be demonstrated macroscopically by adding the serum to the requisite number of drops of the typhoid culture in small tubes, and after thorough admixture setting aside for from 1 to 6 hours in the incubator at 37°C. A positive reaction





is indicated by precipitation of the bacteria in little clumps to bottom of tube and clearing of the mixture.

7. Make inoculations of *Bacillus dysenteriae*, *Bacillus paratyphosus*, and *Bacillus enteriditis* on,

- (a) agar tubes;
- (b) broth tubes;
- (c) litmus milk tubes.

8. Make inoculations of above bacilli and also *Bacillus coli* into,

- (a) dextrose broth fermentation tubes;
- (b) neutral red lactose broth fermentation tubes;
- (c) lactose bile fermentation tubes.

Contrast growth of these bacilli in open and closed arm, the reaction of media, the amount and character of gas production (if any).

## LESSON XIX.

*Bacillus dysenteriae*—*Bacillus paratyphosus*—*Bacillus enteritidis*—*Bacillus pestis*—*Micrococcus melitensis*—*Bacillus mallei*—Cultures of anaerobic bacteria.

1. Compare hanging drop preparations from broth cultures of *Bacillus dysenteriae*, *Bacillus paratyphosus* and *Bacillus enteritidis*.

2. Prepare and contrast smears of these bacilli from the agar cultures. Stain in anilin gentian violet or diluted carbol-fuchsin. Note also reaction to Gram's stain.

3. With the dried blood drops from typhoid fever case furnished, make preparations as for the Widal reaction, substituting broth cultures of these bacilli for typhoid bacillus.

Make notes as to findings at end of 1 hour.

4. Prepare cover glass films from the agar and broth cultures of *Bacillus pestis* and *Micrococcus melitensis* furnished. Stain in Loeffler's methylene blue.

5. In like manner prepare and stain films from the agar and potato cultures of the *Bacillus mallei*.

6. Make inoculations in the usual manner of *Bacillus tetani*, *Bacillus oedematis maligni*, *Bacillus capsulatus aerogenes* (*welchii*) and *Bacillus botulinus* on,







(a) agar tubes (stab) to which .5% formate of soda has been added;

(b) glucose broth tubes.

Treat these cultures by Buchner's method, as described below.

*Anaerobic culture methods.*

Anaerobiosis may be obtained in several ways. One of the most commonly employed methods, particularly when working on a large scale, is to replace the oxygen (air) by hydrogen gas. For culture tubes and plates Novy's jars are very convenient for use with hydrogen.

Another method of producing anaerobiosis is that of Buchner. It consists in the absorption of the oxygen by the use of freshly prepared pyrogallate of potash. This may be carried out in large jars, or in large test tubes, as follows:

(a) In the bottom of a large test tube, place one-half inch layer of sand or absorbent cotton, and then add 1 gramme pyrogalllic acid.

(b) Now place in this test tube one of the inoculated tubes and add 10 cc. of 1% caustic potash solution, taking care not to soil the inoculated tube.

(c) Immediately plug firmly the large test tube with a rubber cork. Pour melted wax or paraffin about the edges of the cork to secure perfect sealing.

The oxygen is quickly absorbed and the bacteria develop in an atmosphere of nitrogen.

## LESSON XX.

*Bacillus tetani*—*Bacillus oedematis maligni*—*Bacillus capsulatus aerogenes*—*Bacillus botulinus*—Cultures of *Tricophyton tonsurans* and *Achorion schonleinii*—Hairs in *tinea tonsurans* and epidermal scrapings in *tinea versicolor*—Animal inoculation (class demonstration).

1. Make hanging drop preparations from the broth cultures of *Bacillus tetani*, *Bacillus oedematis maligni*, *Bacillus capsulatus aerogenes* and *Bacillus botulinus*.

Contrast the motility of these bacilli.

2. Make smears from the broth cultures of each of these bacilli.

After clearing in 10% acetic acid, stain in anilin gentian violet. Note the presence and position of the spores in two former bacilli and in *Bacillus botulinus*.

3. Remove in the usual manner a small portion of the growth of *Tricophyton tonsurans* from the cultures furnished. Tease out on a slide after treatment with 5% caustic potash solution, or with the alcohol-ammonia mixture. Examine with the low and high dry lenses.





4. In like manner examine a preparation from the culture of *Achorion schonleinii* (*Favus fungus*).

Compare this fungus with *Tricophyton tonsurans*.

5. Treat in the same manner the hairs furnished from *tinea tonsurans*. Mycelial threads and spores are seen lying in the root sheath and in the hair shaft.

6. In the same manner tease out the epidermal scales from a case of *tinea versicolor*. Short mycelial threads and spore masses are present.

*Animal Inoculation (Class demonstration).*

(a) Inoculate a guinea pig subcutaneously with .25 cc. of a 72 hour broth culture of *Bacillus tetani*, tearing the tissues slightly at a point of inoculation.

(b) Inoculate a guinea pig subcutaneously with .5 cc. of a broth culture of *Bacillus oedematis maligni*.

(c) Inoculate .5 cc. of a broth culture of *Bacillus capsulatus aerogenes* into the posterior auricular vein of a rabbit. Five minutes after the inoculation, kill the rabbit by a sharp blow on the back of the neck. Keep the body in a room at the temperature of 20°C. and examine after 18 to 24 hours. Gas formation is seen in the veins everywhere, the viscera will be "foaming", and the animal generally emphysematous.

(*d*) Inject 2 large guinea pigs with .2 cc. of tetanus toxin (secured by filtration of broth cultures of *Bacillus tetani* through a Chamberland bougie).

The animals die in from 3 to 7 days.

(*e*) Inject 2 large guinea pigs with .2 cc. of tetanus toxin mixed with .5 cc. of some standard commercial tetanus antitoxin.

These animals should develop no symptoms.







## LESSON XXI.

Examination of animals inoculated with *Bacillus oedematis maligni* and *Bacillus welchii*—Syphilitic tissue.

1. Examine carefully the guinea pig inoculated with *Bacillus oedematis maligni*, noting the extent and character of the oedema. Reflect the skin in the usual manner. Make smears from the oedema fluid for staining. Also inoculate an agar slope and an agar stab tube and two broth tubes with loops of the fluid.

Open up the abdomen and, removing spleen, make smear preparations of the splenic pulp, as described under anthrax. In like manner make preparations from the heart's blood.

Stain these films by methods described for anthrax (page 29).

2. Examine the animal inoculated with *Bacillus welchii*, and then killed and kept 18 to 24 hours.

Make smear preparations from the blood of the various organs.

Stain as with preceding specimens. Capsules are seen surrounding the bacilli.

Inoculations of the blood should also be made on agar slope and stab tubes and on broth as with oede-

ma fluid. All tubes should be incubated at 37°C., the slope agar under ordinary aerobic conditions, the balance under anaerobic conditions. Examine cultures at end of three days and note findings.

### *Syphilitic Tissues.*

3. Mount sections provided, from the liver of a syphilitic foetus stained by Levaditi's method.

Sections are in xylol and should be transferred to slide and mounted in Canada balsam.

The spirochaetae are stained black, the tissues a light yellow, and the reticulum brown.

Levaditi's method for staining spirochaeta pallida in tissues is as follows:

1. Fix small pieces (not more than 1-12 inch thick) of fresh tissue, in 10% formalin for 1 to 3 days.

2. Rinse thoroughly in water and place in 95% alcohol for 24 hours.

3. Transfer to distilled water for several hours (till tissue sinks to bottom of container).

4. Place in 1.5 to 2% solution of nitrate of silver and keep in the incubator at 37°C. away from the light for from 3 to 5 days, changing the silver solution once or twice.

5. Wash in several changes of distilled water and then place for 1 to 3 days in a solution containing





2% pyrogallie acid in 5% solution of formalin in distilled water. Keep in the dark and at room temperature.

6. Dehydrate with graded alcohols, clear with xylol and embed in paraffin. Section and remove paraffin with xylol, from which sections are then mounted in balsam.

## LESSON XXII.

Mouth spirochaetae—Culture of *Spirochaeta pallida*

1. From about the neck of, or from between the teeth remove a little of material which tends to adhere there, and mix with drop of water on slide. Apply a cover glass and examine with high dry and oil immersion objectives. Among other bacteria motile spiral forms are practically always present.

2. Make smears of this material, dry and stain by Loeffler's methylene blue and also by Wright's blood stain (q.v.). Examine under the oil immersion objective and note findings.

3. Place a drop of water on slide, surround by several loops of India ink and then mix together with some of material from teeth, spreading into a thin smear. When dry examine under the immersion objective when the bacteria appear unstained on a dark background; spirochaetae show up well this way.

*Spirochaeta Pallida* (Noguchi).

4. From the ascitic tissue agar culture of *Spirochaeta pallida* furnished, make a preparation like preceding. The spirochaeta appear as white spirals against a dark background.

5. Make a smear in usual manner, dry and stain by Wright's blood stain. The spirochaetae stain faintly blue.







Serum obtained by lightly curetting a chancre or secondary syphilitic lesion of skin or mucous membrane, can be examined by either of preceding methods or by dark ground illumination.

Examine the preparations of *spirochaeta pallida* and mouth *spirochaetae* under the microscopes with dark ground illumination. Contrast size and closeness of spiral turns.

## LESSON XXIII.

Phagocytosis and opsonins.

*Preparation of material to demonstrate phagocytosis and opsonins.*

1. In a sterile centrifuge tube put 10 cc. of 1.5% sodium citrate in normal (.85%) salt solution. Drop into this 12 drops of blood from finger obtained by puncturing finger just back of root of nail after first applying a rubber band above to constrict venous return. Shake thoroughly and centrifuge till fluid is clear, then pipette off the clear fluid above the deposit of corpuscles. Fill up again with normal salt solution, shake thoroughly, centrifuge as before and

be repeated the third time if thought desirable to remove all traces of serum adherent to corpuscles. With a pipette now remove the superficial layers of the sedimented corpuscles, and place in small sterile test tube. This layer will contain most of the leucocytes, mixed of course with many red cells.

2. From a 24 hour agar culture of *Staphylococcus pyogenes aureus* take a loopful of the growth and emulsify thoroughly in a watch glass filled with normal salt solution.

3. In a Wright's pipette collect 8 to 10 drops of blood from finger or ear. Allow to clot and after half an hour centrifuge so as to obtain the clear serum.

The three requisites, viz., washed leucocytes, suspension of bacteria, and fresh blood serum are now ready for use.

4. (a) Draw into a pipette with nipple attachment, a column of the washed leucocytes, to a point marked on stem. Now let in a bubble of air and then draw up a similar column of the suspension of *Staphylococcus pyogenes aureus*. Again let in a bubble of air followed by a column of normal salt solution. Mix these by forcing the contents of the pipette out into a sterile watch glass and drawing it backward and forward into the pipette. When thoroughly mixed draw up into the pipette, seal the again pipette off the clear fluid. The washing may





tip and place in the incubator at 37°C. for 15 minutes.

(b) Repeat this procedure but draw up a column of fresh blood serum instead of the salt solution.

In (a) there are present leucocytes and bacteria; in (b) leucocytes, serum and bacteria.

5. Withdraw pipettes from the incubator, break the seal, and after again mixing the contents as described in 4(a) make smears and stain by Wright's stain.

Examine these smears with the oil immersion objective, taking particular note of the polymorphonuclear leucocytes. Count the numbers of bacteria in first 25 polymorphonuclears seen on slides made from both pipettes.

## LESSON XXIV.

Haemolysis—Noguchi's modification of the Wassermann reaction.

### *Phenomena of Haemolysis.*

(a) A rabbit is immunized against human blood corpuscles by injection intraperitoneally at 5 day intervals of washed human corpuscles. To obtain these 9 parts of blood are poured into tubes containing 1 part 1% sodium oxalate solution and mixed at

once so as to prevent clotting. This mixture is then poured into sterile centrifuge tubes up to 2 cc. mark and normal salt solution added to fill tubes. After shaking, the mixture is centrifuged to sediment the corpuscles, the salt solution poured off and fresh solution added and process repeated. After second or third washing and removal of supernatant salt solution sufficient salt solution is added to fill up to 2 cc. mark. If large tubes are employed the same proportions would hold.

Five injections are usually given the first of about 5 cc., increasing gradually each time till 20 cc. is given at 5th dose. Eight or nine days after last dose the animal is bled into a sterile jar, the blood allowed to clot and kept in the refrigerator for 24 hours to allow the serum to separate. The clear serum is then pipetted off and heated to 55°C. for 30 minutes to destroy complement. The serum contains anti-human (red blood corpuscle) amboceptor. It will keep for some time stored in sterile tubes, but is kept best by absorbing it into filter paper, and drying the paper in a current of cold air. This paper is then cut into strips and stored in sterile dry test tubes in which form it retains its activity for long periods. Such immune serum shows considerable variation in strength in different animals and in exact work it is necessary to determine its haemolytic properties accurately by titration with standard corpuscle suspension in presence of complement.







(b) The blood corpuscle suspension used in prepared as follows: Into a sterile centrifuge tube drop from a capillary pipette 20 drops of normal salt solution and mark with grease pencil the height of fluid. Now fill up the tube with salt solution and drop in 4 drops of fresh blood (normal) from finger. Mix and centrifuge till corpuscles are all precipitated. Remove the salt solution down to corpuscle layer, add fresh salt solution and repeat. After removal of all the salt solution fill up the tube to the pencil mark with salt solution. One drop of this suspension in 1 cc. of normal salt solution constitutes the standard blood corpuscle suspension.

(c) The complement used is obtained from freshly drawn guinea pig blood. If only a small amount is required the blood can be withdrawn under an anaesthetic, from the heart, with a sterile syringe. If large amounts it is best to kill the animal after shaving neck by cutting the neck vessels with a sharp knife. The serum which separates from the clot contains the complement. The serum should not be over 24 hours old when used. .02 cc. of this serum is the standard unit for use with the corpuscle suspension. As it is difficult to measure this amount the serum is diluted with 4 times its volume of salt solution so that .1 cc. of the diluted serum is employed.

Strips of antihuman amboceptor paper cut into unit lengths, suspension of human red blood corpuscles,

diluted fresh guinea pig serum and normal salt solution are furnished.

Set up four small tubes. In all, place 1 drop of corpuscle suspension using pipette with rubber nipple. To Nos. 2, 3 and 4 add .1 cc. of complement. To all add 1 cc. of normal salt solution. To Nos. 1 and 2 add a strip of the amboceptor paper, to No. 3 a portion cut in half. Shake the tubes, incubate at 37°C. for two hours and note results.

*Noguchi's modification of the Wassermann reaction for syphilis.*

In addition to the materials required for haemolysis of human blood corpuscles as just recorded, this method requires syphilitic antigen and the serum of the suspected individual. For control purposes the serum of a normal individual and that of a known syphilitic are desirable.

The antigen used is not a special syphilitic substance but a tissue extract. The antigen employed is an alcoholic solution of the acetone insoluble portion of an alcoholic extract of guinea pig heart (though human or bovine heart or liver can be used equally well). In use .1 cc. of the alcoholic solution is added to .9 cc. of salt solution and .1 cc. of the mixture used for the tests. In this amount of properly made antigen there should be 5 antigen units, i.e. this amount of antigen would require 5 complement units to completely saturate it. The antigen





must be tested to show absence of haemolytic or anti-complementary properties.

The serum of the patient if syphilitic will contain an amboceptor active against this antigen. The serum is obtained by drawing 20 to 25 drops of blood into Wright's pipettes, allowing same to clot and then separating the serum by use of centrifuge. Larger amounts can be obtained by drawing blood by syringe from vein. The serum should be inactivated (have its complement destroyed), by heating to 55°C. for 30 minutes. The serums of normal and syphilitic controls are obtained in same manner and inactivated.

All the various constituents must be carefully standardized and used in proper proportions. In making the tests 2 units of complement (.04 cc. of fresh guinea pig serum) and 2 units of antihuman amboceptor are employed.

The test depends on fact that complement is non-specific and having united with one amboceptor to attack the antigen *is not available* for union if later a second amboceptor and antigen are added, hence there is no interaction between the last added antigen-amboceptor. This is the Bordet-Gengou phenomenon of deviation of the complement.

In making the tests a double set of tubes for each serum to be tested is employed. Drops are added by sterile capillary pipettes.

Normal Serum		Suspected Serum		Syphilitic Serum	
Tube 1	Tube 2	Tube 3	Tube 4	Tube 5	Tube 6
4 drops normal serum . . . . .	Do. . . . .	4 drops suspected serum. Do. . . . .	4 drops syphilitic serum. Do. . . . .		
	.1 cc. Antigen		.1 cc. Antigen		.1 cc. Antigen
.04 cc. complement . . . . .	Do. . . . .	Do. . . . .	Do. . . . .	Do. . . . .	Do. . . . .
1 cc. normal salt solution. Do. . . . .	Do. . . . .	Do. . . . .	Do. . . . .	Do. . . . .	Do. . . . .
Incubate all tubes at 37°C. for one hour, then add					
1 drop human blood corpus. Do. . . . .	Do. . . . .	Do. . . . .	Do. . . . .	Do. . . . .	Do. . . . .
2 units Anti-human Amboceptor . . . . .	Do. . . . .	Do. . . . .	Do. . . . .	Do. . . . .	Do. . . . .

Shake well and incubate at 37°C. for 2 hours. Remove and read. Tubes 1, 2, 3 and 5 should show complete haemolysis. Tube 6 should show no haemolysis, while presence or absence of haemolysis in tube 4 determines if reaction is negative or positive.











## PART II.

## PREPARATION OF MEDIA.

## CULTURE MEDIA.

For a study of the general biological characters of bacteria, growths in pure culture are required. For this growth certain nutrient materials are necessary, the nutrients varying more or less for the various species of bacteria. For the pathogenic bacteria, media approximating to the nutrients of the body are most employed. Desirable characters in culture media are clearness and ease of preparation and sterilization. The methods of preparation of the culture media in common use only will be given.

*Broth* or *bouillon* is much employed itself and as the basis for nutrient jellies, as agar and gelatine.

One pound of lean beefsteak, freed from all fat and connective tissue bands, is chopped up in a sausage machine. One litre of distilled water is added and the mixture is allowed to stand in the refrigerator 12 to 24 hours, and is then filtered through cotton flannel, and distilled water added to make up to one litre. Place in a container with 10 grammes of peptone (Witte's) and 5 grammes of common salt. The mixture is heated in a water bath or steam sterilizer

for 30 minutes to dissolve these, and reaction is then adjusted by addition of normal sodic hydrate solution. The standard reaction is  $+1$ , (meaning that to completely neutralize it would be necessary to add 1 cc. of normal soda to each 100 cc. of broth), and the amount of normal soda required is determined as follows:

To 10 cc. of the broth add 50 cc. of recently boiled distilled water and 1 cc. of phenolphthalein solution (.5 gm. phenolphthalein in 50 cc. each of alcohol and water) and bring to a boil. From a burette add drop by drop decinormal sodic hydrate till a pale but decided pink color develops. The number of cubic centimetres of decinormal soda required to so neutralize will be the amount of normal sodic hydrate to be added per 100 cc. of broth, to completely neutralize. This amount less 1 cc., for each 100 cc. of broth is then added, and the mixture steamed for 15 minutes in the sterilizer or boiled over flame for 2 to 5 minutes. Make up any loss by evaporation and allow to cool. Filter through moistened filter sheets. Titrate again and adjust reaction to  $+1$  by addition of normal soda or normal hydrochloric acid as may be required.

The broth is then steamed for 15 minutes, cooled and filtered into flasks or tubes and complete sterilization effected by steaming for 30 minutes on three successive days, or by placing in autoclave at  $120^{\circ}\text{C}$ . for 15 minutes.

Instead of beef steak, Liebig's meat extract (Lemco) is now much employed, 5 grammes of this extract being dissolved in 1000 cc. of water, with 10 grammes of peptone and 5 grammes of salt. The after treatment is the same as before described.





Broth so made is equal in all respects to that made with meat infusion for all ordinary purposes, though for water analysis the meat infusion broth is preferable.

*Glycerine broth* is ordinary broth to which 2 to 6% of pure glycerine is added, before sterilization.

*Sugar broths* are ordinary broths to which 1 or 2% of the pure sugars have been added before sterilization. Autoclave sterilization at 120° for 15 minutes is preferable for sugar containing media. If amount of acid formation by bacteria is to be estimated on sugar broths, these should be completely neutralized.

*Neutral red lactose broth* is ordinary broth to which 2 grammes of lactose and 1 cc. of a .5% aqueous solution of neutral red per each 100 cc. of broth have been added before final sterilization.

*Gelatine.* To 1000 cc. of the stock broth or of broth ingredients add 100 grammes of gelatine ("gold label") cut up into small pieces. Dissolve carefully over a water bath, and adjust reaction with normal sodium hydrate as described under broth. Place in a flask, cool down to 60°C., and add the whites of two eggs dissolved in 50 cc. of water. Steam for 1½ hour in the sterilizer and filter through moistened filter sheets.

Now restore to original volume and adjust reaction (+1), tube and sterilize. Autoclave sterilization at 120°C. for 15 minutes is best.

*Agar.* Cut up 15 grammes of the best agar fibre and allow to soak in water. Pour off water and place in a flask with 1000 cc. of stock broth or of broth ingredients, and dissolve by heating in the autoclave, up to  $120^{\circ}\text{C}$ . After removal from the autoclave, adjust reaction to +1 with normal sodic hydrate, cool to  $60^{\circ}\text{C}$ ., and add the whites of two eggs dissolved in 50 cc. of water. Cook in the autoclave for 10 minutes at  $110^{\circ}\text{C}$ ., and using a warming funnel, filter through moistened sheets. (Chardin's agar filter paper is desirable for this purpose). As a rule the agar runs through rapidly. If it tends to clog, immediately replace by fresh paper, keeping the unfiltered solution hot. Restore to original volume, adjust reaction to final point desired and sterilize.

Several agar modifications are used such as sugar agars and glycerine agars which are made like corresponding broths. *Blood agar* is usually made by smearing sterile human or rabbit's blood over the surface of slope agar tubes, or by adding same to tubes of liquefied agar kept at  $42^{\circ}\text{C}$ . and allowing these to set as slopes or in plates as required. *Ascitic agar* is made by this last method, usually one part of ascitic fluid being used to two parts of melted agar.

*Drigalski and Conradi's medium* which is employed for detection of typhoid bacilli is made as follows:

(a) To one litre of meat infusion is added 10 gm. of Witte's peptone, 10 gm. neutrose and 5 gm. of







sodium chloride, and the whole boiled for 1 hour, filtered and volume restored to 1 litre. To this is then added 30 gm. of washed agar fibre and solution effected in the autoclave at  $110^{\circ}\text{C}$ . for 1 hour. Normal sodic hydrate is then added till mixture reacts faintly alkaline to litmus and it is then autoclaved at  $110^{\circ}\text{C}$ . for 20 minutes and filtered through moistened filter paper using the warming funnel. The filtrate is then autoclaved for 15 minutes at  $120^{\circ}\text{C}$ . to complete sterilization.

(b) 130 cc. of litmus solution (Kubel and Tie-mann's) is boiled for 10 minutes and 15 gm. of pure lactose added and again boiled for 15 minutes.

The hot litmus lactose solution is mixed with the sterilized fluid agar and sufficient normal sodic hydrate added to give a distinct bluish tinge to the froth when flask is agitated. Then add 10 cc. of fresh solution of 1 to 1000 crystal violet in sterile distilled water.

Store this in small flasks so as to require minimum of heating to melt. In using pour melted mixture into plates and allow to set, the infecting material being then rubbed over surface.

*Blood serum.* Blood from cattle is collected at the slaughter house, in well stoppered sterile litre jars. The blood is best collected after it has run for some time from the cut vessels, as it lessens the danger of

contamination. The jars are then set on ice for 24 to 48 hours, and the serum which collects is syphoned off, into sterile flasks. The presence of some haemoglobin in the serum does not interfere with its value, though it darkens the media. In fact, this dark blood serum is preferable for growth of many bacteria.

Several culture media may be prepared from this serum.

(a) Loeffler's serum mixture. To 3 parts of the serum add 1 part of 1% dextrose broth. Run into sterile test tubes and then place these tubes properly slanted in the serum coagulator, and expose them for  $2\frac{1}{2}$  to 3 hours to a carefully regulated temperature of from 90 to 95°C. This firmly sets the tubes. Afterwards they may be steamed for 30 minutes in the sterilizer on 3 successive days, keeping the steam vent partially open.

(b) Hiss' serum water media. To 1 part of the beef serum add two parts distilled water. Sterilize in steamer on three successive days for 30 minutes. To this media sterile litmus solution and various sugars can be added if desired. Instead of the water, broth may be employed. Ascitic, pleuritic or hydrocele fluids may be substituted for the beef serum. It is advisable to test the coagulability of the serum water mixture as above outlined before sterilization, as some serums require greater dilution.





*Milk.* Fresh separated milk (readily obtained from creameries) should be used, as otherwise the cream is troublesome. Sterilize by steaming for 30 minutes on 3 successive days.

*Litmus milk* is made by adding sufficient litmus solution to milk (neutralized is necessary) to give a distinct blue tinge. The milk is then sterilized in the usual manner.

*Potato.* Select fair sized smooth potatoes and wash thoroughly. Slice off the end, and, with a cork borer slightly smaller than the test tubes to be used, cut out cylinders from  $1\frac{1}{2}$  to 2 inches long. Cut these cylinders diagonally across and allow them to soak in a weak ( $\frac{1}{10}$  of 1%) solution of sodium hydrate for one hour. Now place in the bottom of the test tubes to be used a small plug of moistened absorbent cotton, and then put in each, one of the potato plugs. Sterilize in the autoclave for 30 minutes at  $120^{\circ}\text{C}$ .

*Bile.* This consists of fresh ox-bile to which 1% of Witte's peptone is added, and then boiled and filtered through cotton wool. The filtrate is then sterilized by steaming for 30 minutes for 3 days. A 10% aqueous solution of fresh dried ox-gall can be used instead of fresh bile.

*Lactose bile* consist of above plus 1% lactose. This medium is now extensively employed in water analysis.

*Egg Media (Dorset).*

Wash perfectly fresh eggs in 5% carbolic acid solution, dry on sterile towel and break into a sterile flask. For each egg add 20 cc. of sterile broth and mix thoroughly. Run the mixture into sterile tubes, place in slanting position in serum inspissator and coagulate at temperature of 75 to 80°C. retained for 4 or 5 hours. Sterilization may be completed by repeating the heating in inspissator for 3 days, but better results are obtained by steaming in sterilizer for 30 minutes for 3 days, keeping the steam vent partially open. For *glycerine egg media* 5% of glycerine is added to the mixture before tubing.

*Peptone solution* of Dunham. To 100 cc. of distilled water add 1 gramme Witte's peptone and .5 gramme of common salt. Boil together for 15 minutes, filter into flasks or tubes, and sterilize these for 30 minutes in the steam sterilizer on 3 successive days.

*Preparation of Utensils.*

*Test tubes.* If the tubes are new, rinse several times in water and then swab out with 10% nitric acid. Thoroughly rinse through water and allow to dry on a draining rack.

Plug these tubes with raw cotton and sterilize in the dry air oven at 150°C. for 1 hour.







Tubes which contain old culture media or growths should, after removal of the cotton stopper, be placed in 1 to 500 bichloride of mercury for 24 hours. The media should then be removed, the tubes rinsed out with water and then boiled for one hour in water with some hard soap added. The tubes are then rinsed in warm water and cleansed with brush, again rinsed and treated like new tubes.

*Slides.* New slides need no particular treatment before use, but if greasy should be kept in alcohol. Slides used, should be placed in "waste" alcohol for 24 hours, so as to free the cover glasses, and then briskly rubbed and rinsed in water. Boil for 15 minutes in soap and water. Rinse in warm water and then in 5% nitric acid, for 15 minutes. Rinse again in water and keep in alcohol.

*Glassware.* Thorough rinsing in water suffices for the cleaning of all glassware not contaminated with culture media, or like material. When so contaminated boil in soap and water and then rinse through water and 5% nitric acid, followed by repeated rinsing in water. Modifications in this process will be required to remove special forms of soiling. Sterilization is effected in the hot air sterilizer at 150°C. for one hour.

*Instruments.* Knives, scissors, forceps and hypodermic needles are best sterilized by boiling from 5

to 10 minutes, after protecting any cutting edges by wrapping absorbent cotton about them. Sterilization may also be effected in 5% carbolic acid solution exposing the instruments to its action for at least  $\frac{1}{2}$  hour. Sterilization is readily and rapidly effected, by placing the instruments in the flame, but of course this rapidly destroys the instrument. Boiling is certainly the most efficacious method for daily use. Hypodermic needles after sterilization may be kept in a 1% sodium carbonate solution, well covered from air.





## PART III.

## BACTERIOLOGICAL ANALYSIS.

## WATER ANALYSIS.

Bacteriological water analysis is of great importance from the public health viewpoint, as it affords the readiest and most accurate analytical means of determining the presence and extent of infection of water with bacteria of intestinal origin, and hence indicates the disease carrying possibilities of water. It is also used as index of the bacterial efficiency of various methods of water filtration or chemical treatment.

Care should be exercised in collection to obtain a specimen which will be an average sample of the water. The water should be collected in cleansed sterilized bottles, stoppered with a closely fitting ground glass cork. Care must be exercised in collecting not to soil the neck of the bottle or cork, keeping in mind that the fingers will readily soil these. The neck of bottles, previous to and after collection, should be carefully protected with rubber tissue. The samples should be sent at once for analysis, as even two to three hours in warm weather may change, to a considerable extent, the bacterial content of the water. This is of particular importance where numbers of bacteria present are to be deter-

mined. If there is any delay before an analysis can be made, the sample should be carefully packed in ice.

In making a water analysis a knowledge of the source of sample is almost a prime necessity for a proper interpretation of findings. If a water is from a suspicious source it may be necessary to make dilutions of sample with sterile water before starting the determination. Again where a water is subject to slight or occasional pollution only, amounts up to 100 cc. or even a litre may need to be used. In ordinary practice 4 to 6 ozs. (100 to 150 cc.) are sufficient for bacterial analysis.

Counts of numbers of bacteria present are especially useful in determining efficiency of filtration or other treatment, contrasts being made between treated and untreated samples. Such counts are also useful in making a series of tests of a public water supply so as to determine effect of winds, currents and climatic conditions. Again, such counts are valuable in a systematic analysis of a lake, river or other proposed source of public water supply, in assisting in the determination of best location for an intake.

Tests for intestinal bacteria of which *Bacillus coli* group is the standard, are of great importance as indicating faecal pollution of water and, if traceable to human sources, the possibility of water carried disease resulting from use of such water.







Tests for *Bacillus coli* should be both quantitative and qualitative, as its abundance rather than mere presence is the point of importance. Streptococci and *Bacillus enteriditis sporogenes* are faecal bacteria of secondary importance which may be looked for in water analysis. The former is of decidedly more import than the latter.

### *Methods of Examination.*

*Bacterial counts* or estimation of numbers of bacteria in water. There is no method yet known by which all the bacteria in water can be accurately estimated. By making examinations of samples under standard conditions as regards media, time and temperature of incubation and amount of water added, it is possible to obtain data for comparison.

Agar media with +1 reaction is standard medium and should be incubated at 37°C. and 20°C. for 1 and 3 days respectively, 1 cc. of water being used in making plates. The plates held at 37°C. give a better indication of the numbers of bacteria probably related to pathogenic forms, while those at lower temperature are of particular value in estimating efficiency of filtration methods or of other modes of treatment of water.

The procedure in making the water plates is as follows: After shaking the sample bottle for 25 times, withdraw 1 cc. of the water with a sterile pipette and place in Petri dish. Add 10 cc. of lique-

fied agar held at 42°C. and thoroughly mix with the water. If the sample is suspected of having a bacterial content of over 200 per 1 cc., the sample is suitably diluted, e.g., by adding 1 cc. of the water to a tube containing 9 cc. of sterile water and 1 cc. of this dilution then used for each plate. At least two plates should be made and these plates after setting are incubated, one at 37°C. and other at 20°C., for 1 and 3 days respectively, when colonies which develop are counted, using a suitably ruled glass plate with dark background or a Pakes disk, and a magnifying hand lens. On the plates at 37°C. spreading colonies are often troublesome but can usually be overcome by using a porous earthenware cover for the Petri dish.

#### *Test for Bacillus coli.*

The standard medium recommended by the Laboratory Section of the American Public Health Association for the growth of *Bacillus coli* from water is lactose bile. This medium has a slight inhibitive effect on *Bacillus coli* but will readily grow all active types, including all that are of recent intestinal origin, and hence the ones of main import from sanitary viewpoint. Dextrose broth and especially dextrose liver broth (liver infusion substituted for meat infusion) will develop all gas forming bacteria in water, including the attenuated types of *Bacillus coli*.





In many Laboratories, neutral red lactose broth is employed and is nearly equal in efficiency to lactose bile, though it will develop some non-colon gas forming bacteria, while the bile medium inhibits practically all such except *B. welchii*.

Using lactose bile, the procedure for an unknown water is to add 1/10, 1 and 10 cc. amounts of the water to this medium in fermentation tubes and to incubate for 2 days at 37°C. If no gas has developed in this period *B. coli* is absent. If gas develops these amounts afford a rough quantitative estimation of the numbers of *B. coli*. This test is presumptive only, but unless there is special reason to do so, isolation and study of the gas forming bacillus is not necessary, as experience has shown that some member of the *B. coli* group is accountable for gas formation in over 95% of such findings.

With a known water departure from the above quantities may be required. Thus with a much polluted water amounts down to 1/10000 cc. may only show absence of *B. coli*; while good waters may show absence of *B. coli* in amounts of 100 cc. or more. Modifications have thus to be made to meet the character of water to be examined and to give a rough estimate at least of numbers of *B. coli*, if present at all.

In the use of neutral red lactose broth, the same general procedure is followed as above. Presump-

tive indications of presence of *B. coli* are gas formation and change from red to yellow of the media in closed arm of fermentation tube. Isolation of the gas forming bacterium shows in 90% of instances the presence of a member of *B. coli* group.

With dextrose broth or liver broth as culture medium a larger number of gas forming bacilli develop and isolation of these and study of their morphological and fermentative characters are necessary before pronouncing them to belong to *B. coli* group.

To isolate *B. coli* from the fermentation tubes, plate cultures from suitable dilutions should be made, using lactose litmus agar or Drigalski-Conradi medium. The colonies which develop (or a reasonable number of them) are then subcultured and their morphological and fermentative reactions studied.

The characteristic features of a member of the *B. coli* group are as follows: *Bacillus* with rounded ends, non-spore forming, Gram negative, fermenting dextrose and lactose, not liquefying gelatine in two weeks, nitrate reducing. Nearly all members of the group produce indol.

Apart from inspection of sources of water there is no means of differentiating between *B. coli* of human and animal origin.

#### *Test for Bacillus typhosus.*

In the majority of cases where suspicion of causing typhoid fever falls on a water supply, sufficient







time has elapsed for the disappearance of the typhoid bacillus from such water unless the infection is constant. *B. coli* being more resistant thus proves a better indicator of the disease carrying possibilities of the water and is always present if typhoid bacilli gain entry. Hence the absence of *B. coli* may be considered a positive indication of absence of *B. typhosus*.

The typhoid bacillus grows even more readily on lactose bile than *B. coli*, hence this medium is used to make preliminary cultivations from water as in ordinary *B. coli* tests. After two days' incubation if gas is formed, plate subcultures are made on the Drigalski-Conradi medium (or on other special typhoid media). The blue colonies which develop on incubation for 18 to 24 hours are then subcultured and examined in detail (see Lesson XVIII).

#### *Test for Streptococci.*

Most intestinal streptococci develop poorly on bile media, hence if these bacteria are to be looked for dextrose broth or lactose neutral red broth should be employed, the former being preferable. The streptococci will be found in the bend of the fermentation tube after two days' incubation and are best detected by transfer of a loop or drop of culture fluid from this part and its examination unstained. If desired, streptococci can be isolated on ordinary agar plates from the fermentation tube, incubating plates for

two days and examining small pin point colonies which develop. The presence of streptococci is held to indicate recent faecal contamination from human beings or horses.

*Tests for B. enteriditis sporogenes.*

This bacterium develops poorly on lactose bile, hence if tests are to be made for this bacillus dextrose broth or liver broth should be employed for preliminary cultivations from the water. The procedure is otherwise the same as for *B. coli*. On these media in 24 hours if *B. enteriditis sporogenes* is present there will be abundant gas formation with an offensive odor and strings of bacilli with large spores will be found on examination of the fluid from the bend of fermentation tube.

To isolate this bacillus, the 24 hr. old fermentation tube cultures should be heated for 1 hour at 80°C. and 1 cc. of the heated culture then transferred to milk tubes. The milk is then incubated anaerobically for 24 hours, when the milk will be curdled, the curd riddled with gas holes, and have a rancid (butyric) odor.

### SEWAGE ANALYSIS.

Bacterial analysis of sewage is based on same principles as for water analysis, the *B. coli* group being the bacteria of special import.





In testing untreated sewage, dilutions of the sewage in sterile water are made ranging from 1 in 10,000,000, up. Such dilutions are made by transferring 1 cc. of the sewage to a bottle with 99 cc. of sterile water. After thorough admixture, 1 cc. of this is transferred to second bottle with 99 cc. of sterile water and the series continued until desired dilution is obtained. Treated sewage will require less dilution. Bacterial counts and tests for *B. coli* or other faecal organisms are made as already described under water analysis.

#### MILK ANALYSIS.

A bacteriological examination of milk may be required to determine if milk supply of the city or town is below a maximum bacterial standard, and to determine the presence or absence of disease producing bacteria. As milk is an excellent medium for bacterial growth, especially of lactic acid producing species, the temperature at which milk has been kept after milking and the time which has elapsed before sampling are very important elements in the analysis.

Special care must be taken to keep samples well iced from time of collection to examination and to make examinations as quickly as possible after collection.

The principles which govern milk analysis are similar to those for water analysis. Bacterial counts

are however of much more importance while tests for intestinal (animal) bacteria are subsidiary. *Bacterial counts* are made by same methods as for water but samples will always require dilution from 10 to 100,000 times according to age, and temperature at which sample has been held previous to collection.

It is advisable to keep the set of plates at 37°C. for two days and those at 20°C. for five days before reading.

A more rapid method of estimating the number of bacteria is that of Slack. The milk is thoroughly mixed and the special tube (holding about 2 cc.) is filled, stoppered, and then centrifugalized for 10 minutes at 2500 revolutions per minute. The stopper at inner end of tube is withdrawn and cream removed and milk then poured out. The open end of tube being held downward, the stopper at outer end with adherent sediment is now removed and sediment smeared with a drop of water over an area on slide covering 4 sq. cm. (2 x 2 cm.). This smear is then carefully dried, fixed and stained with methylene blue. Such a smear will show the approximate number and variety of bacteria present and the cellular constituents including any pus. A count of the bacteria in a field of the 1/12 oil immersion objective multiplied by 10,000 gives approximately the number of bacteria per 1 cc. of milk. This method is







valuable in routine work as the whole time occupied would average less than 30 minutes for a single specimen, and where a series are being examined 20 specimens can be examined per hour. Those specimens which show a high bacterial content can then be plated in ordinary manner. This method also gives a fair estimate of the numbers of pus cells (leucocytes) in milk and the presence or absence of streptococci. The numbers of pus cells present per 1 cc. milk can be estimated by multiplying the average number per field in 12 fields of the 1/12 oil immersion objective by 20,000. Little stress can be laid on numbers of pus cells in milk unless these are accompanied by distinct chains of long streptococci, when mammitis will be found in some of cattle supplying milk.

As the numbers of bacteria in fresh milk (or in milk cooled at once after milking below 50°C. and held at or below this point for not more than 30 hours), bears a close relationship to the amount of contained "dirt", a simple machine has been devised to collect this sediment. A thin circular absorbent cotton pad 1 inch in diameter is fixed over a wire screen held at bottom of a container holding 1 pint and fitted with tight cover with a vent connected with a rubber bulb. The milk is well stirred and the container filled quickly with milk, cover fitted and milk forced through the pad. This requires about 30 seconds, and all particles are held on the absorbent

cotton, which is removed and can be kept as an exhibit. Experience has shown that fresh milk with much sediment shows a high bacterial content, and as this test takes less than two minutes and can be performed by any intelligent person, it ought to prove a valuable method of testing fresh market milk.

*Tests for faecal bacteria*, especially *B. coli* group.

These are made in same manner as like tests for water, the amounts employed ranging from 1 cc. down to 1/10000. In ordinary market milk representatives of the *B. coli* group, especially *B. lactis aerogenes* or *acidi lactici* (Hueppe) are found in 75% of the samples in 1 cc. amounts. These bacteria come from manure and stable dust and like sources and are seldom of human origin.

*Tests for pathogenic bacteria.* Tests for typhoid and diphtheria bacilli are practically valueless, for by time suspicion is directed against the milk, the milk actually at fault has long before been consumed and the milk available unless exposed to similar infection will prove negative.

*Tests for tubercle bacilli.* Microscopic tests are very unsatisfactory as bacilli may be readily overlooked, or may be so closely imitated by other acid-fast bacteria that the examiner does not care to give a positive opinion. If tests are to be made at least 50 cc. of milk should be used. This is best diluted





with an equal bulk of sterile water and heated to 60 to 65°C. for 10 minutes to break up the fat globules and clumps. The heated milk is then centrifuged for 10 minutes at 1200 revolutions and the sediment used to smear slides which are stained by the Ziehl-Neelsen process.

More accurate results are obtained by injecting 5 cc. of the sediment obtained by a similar process but without heating, subcutaneously into guinea pigs. Animals which die inside two months are carefully autopsied and examined for tubercular lesions. Animals still alive at end of two months period can be injected with crude tuberculin (usually 2 cc.), when the tubercular pigs die inside 24 hours, the healthy pigs remaining unaffected. The pigs which die must of course be carefully autopsied.

Tuberculosis in cattle is best detected by careful veterinary examination and tuberculin testing.

N.B.—For fuller details of methods of analysis of water, sewage and milk refer to the Standard Methods of Analysis as adopted by the Laboratory Section of the American Public Health Association.

## PART IV.

## CLINICAL MICROSCOPY AND DIAGNOSIS.

## SECTION I.

## URINE.

The sample should be taken from the entire quantity passed in 24 hours, care being taken to preserve urine in a cool place and thoroughly clean container. If such a sample is not available select one passed some hours after a meal. Do not use the morning's urine for routine examination, as in this urine least evidence of disturbance of the renal functions will be found. Samples should be examined as fresh as possible, as putrefaction soon occurs when urine is allowed to stand. A crystal or two of thymol tends to prevent this, and does not destroy the efficacy of any of the coarser chemical or microscopic tests, but should not be employed unless necessary.

*Methods of Examination.*1. *Physical examination.*

(a) Note the *quantity* passed in 24 hours. This is important in calculating the actual daily excretion of any normal or abnormal urinary constituents, as well as determining whether oliguria or polyuria exists.







(b) Note the *color, odor*, the presence and character of any *turbidity* or *sediment*. Various changes in color and clearness are found both in normal and abnormal urines. These changes in normal urine depend upon the reaction, the amount of the solid constituents or upon the onset of putrefaction. The color of the urine depends on amount excreted, the amount of urinary pigment, the presence of such constituents as pus blood or bile, or the precipitation of urates or phosphates.

(c) Take the *specific gravity*. By this we get particularly in a 24 hour specimen, a good indication of the amount of solids in the urine. The usual formula given is to multiply the last two figures by 2.2 and so obtain the number of grammes of solids per litre.

## 2. *Chemical tests.*

(a) Take the *reaction* with litmus paper. The reaction is important in carrying out certain albumin tests. It may also indicate the nature of a sediment. Thus if an acid urine is turbid with a brick dust sediment, this is due to presence of urates which can be confirmed by warming the urine when deposit redissolves. If the urine is alkaline with a white flocculent or amorphous deposit, this is due to earthy phosphates, the turbidity becoming more marked on heating and clearing on carefully acidifying sample with dilute acetic acid.

While urates and earthy phosphates can be dissolved in this manner, *it is best if the urine be at all turbid to filter through several sheets of filter paper, before the application of any test.*

(b) Examine the sample for *albumin*.

1. Use the heat and nitric acid test.

In applying this and the cold nitric acid tests, the urine, if not acid, should be acidified by careful addition of dilute acetic acid. The urine should be heated to boiling, any changes being noted. If specimen becomes clouded or a distinct precipitate forms, two or three drops of nitric acid should be poured down side of tube. If albumin is present the cloudiness remains or intensifies, while cloudiness due to precipitation of phosphates clears.

2. Apply the cold nitric acid test.

Place  $\frac{1}{2}$  inch layer of nitric acid in tube and carefully overlie this with one inch of urine. In presence of albumin a white ring forms at line of junction of two fluids, faint with traces of albumin, deepening with increase in amount.

Simon advises an excellent modification of the usual application of this method. 20 cc. of urine are placed in a conical urine glass, and 6 to 10 cc. of nitric acid are added through a pipette, passed to bottom of glass. At point of junction of the two fluids we have the formation of the usual contact ring in the presence of albumin. This ring is usually colored at bottom, rose to brick red, from the normal urinary pigment.





If indican be present in excess, we have a violet to black ring. In biliary urinary we have the usual contact of colors (acid must contain nitrous acid as well). On standing 5 to 10 minutes, high up in the urine a white band appears, very faint normally but distinct with slight excess of urates or uric acid. If urea be present in larger amount than 25 grammes per litre, it slowly crystallizes out on side of urine glass.

3. Use saturated aqueous solution of salicyl-sulphonic acid, adding the reagent drop by drop. Cloudiness to a dense white precipitate indicate amount of albumin present. Mere traces of albumin are detected with this reagent.

4. Use Esbach's reagent (Picric acid 10 gms., Citric acid 20 gms., boiling water 1000 cc.) with Esbach's albuminometer. After mixing, set aside for 24 hours before reading. Sedimentation can be rapidly effected by use of centrifuge and special tubes.

For details as to fallacies in these methods, and for the differentiation of the various albumins and albumoses a work on urine analysis should be consulted.

Chemical tests for blood and pus are rather unsatisfactory, microscopical examination being superior, especially for small amounts. If blood corpuscles are disintegrated tests for occult blood (q.v.) can be employed.

(c) Apply the tests for *sugar*.

In the presence of albumin the urine should first be boiled and then filtered.

1. Use Fehling's test, the cupric sulphate and alkaline solutions being kept in separate bottles, equal parts being mixed for use. 10 cc. of mixture are reduced by .05 gm. sugar. The composition is as follows:

(a) Dissolve 34.64 gm. of pure crystallized cupric sulphate in warm distilled water and make up to 500 cc.

(b) Dissolve 170 gm. crystallized Rochelle salts in warm distilled water, filter well, add 50 gm. of pure caustic soda and after solution is effected make up to 500 cc.

In making the test, boil the Fehling's solution and urine in separate tubes and then add to the Fehling's solution about one-fourth its bulk of the urine and, if necessary, heat to boiling point again. Reduction to red or yellow ought to occur at once in presence of glucose.

To apply this test quantitatively, place 10 cc. of Fehling's solution, diluted with 40 cc. of water, in a porcelain capsule and bring to a boil. Add from a burette drop by drop the urine diluted 5 to 10 times according to amount of sugar suspected, till all blue color is completely discharged, keeping the solution at boiling point. The amount of diluted urine required will contain .05 gm. sugar.







2. Apply the fermentation test by adding to urine a small amount of fresh yeast and placing in a fermentation tube in a warm place for 12 to 24 hours. Gas formation is an evidence of presence of sugar.

The amount of sugar can be approximately estimated by this method with Einhorn's saccharometer. As this only reads up to 1%, the urine should be diluted 5 to 10 times with water, if sugar be present in any quantity.

3. Apply the phenyl-hydrazin test. 10 cc. of the urine is boiled in a water bath for 30 minutes with 3 or 4 gms. of acetate of soda and .3 to .5 gm. of phenyl-hydrazin hydrochloride. The tube is cooled by plunging into cold water and a microscopic examination made of sediment for phenyl-glucosazone crystals. This is a delicate test.

*Lactose* if present will reduce Fehling's solution but more slowly than glucose. With the phenyl-hydrazin test the crystals are of different character, while the fermentation test is negative.

(d) Test for *acetone*.

This test should be made with all urines containing glucose, in urine from febrile cases and comatose states.

1. Modified nitroprusside test. To 5 cc. of urine add a small crystal (grape seed size) of sodium nitroprusside and allow to dissolve. Acidify with 5

drops of glacial acetic acid, shaking the tube, and overlies with 20% solution of strong liquor ammoniae. At the line of junction a violet ring deepening to dark purple develops inside 5 minutes. The rate of development and intensity of coloration roughly indicates amount of acetone present. No reaction occurs with normal urine.

2. The iodoform test. Mix equal amounts of the urine and liquor potassae and add 3 drops of aqueous iodine solution. Shake and note if odor of iodoform develops or if on standing iodoform crystals are deposited.

This test is best applied to the first distillate from a sample of urine, the acetone rapidly volatilizing and being carried over in first portions of urine in distillation.

### 3. Test for *diacetic acid*.

This test should be made with all urines containing glucose, in urine from pregnant or puerperal women, in cases of serious liver disease and in comatose states.

The test (Gerhard's) is made by adding to urine drop by drop a 10% aqueous solution of the perchloride of iron. A precipitate of phosphates forms, but on adding a few drops more a deep wine red color develops in presence of diacetic acid. The color is best brought out by filtering out the phosphates.





For control, a second tube of urine should be boiled for 2 minutes and then tested. The acid being volatile, no reaction is given.

#### 4. Test for *bile pigment*.

The color of the urine, especially the color of the froth which so readily forms on urine containing bile, is sufficient indication of samples to be tested for bile pigment.

1. The fuming nitric acid test. This may be applied as a ring test as for albumin, but develops better on filtering 15 or 20 cc. of urine through white filter paper and then applying a drop of the fuming acid to the surface of the filter sheet when the play of colors is readily noted if bile be present.

2. The iodine test. Overlie urine in a test tube with 5 cc. of tincture of iodine (or aqueous iodine solution) diluted 10 times with alcohol. An emerald green ring develops at the line of junction between the two fluids in presence of bile. This is a delicate test.

#### (g) Test for *indican*.

An increase in indican is indicated by the purplish to black coloration of the contact ring in applying the cold nitric acid test for albumin. To obtain approximately the amount present mix equal amounts of urine and Obermayer's reagent (2 cc. of 10% aqueous solution of ferric chloride to 98 cc. of hydro-

chloric acid) and add 2 or 3 cc. of chloroform; shake and allow to stand till chloroform settles. Normally this layer is faintly tinged blue, the color deepening as indican increases.

(h) Test for Ehrlich's *diazo-reaction*.

This often gives information of value in suspected typhoid, miliary and advanced pulmonary tuberculosis and occasionally in other febrile affections.

Two stock solutions are kept from which the test solution is made when required for use.

- (1) Sulphanilic acid, 1 gm.  
Hydrochloric acid, 5 cc.  
Distilled water, 200 cc.

- (2) Sodium nitrite, .5 gm.  
Distilled water, 100 cc.

The test solution is made by adding 1 cc. of the nitrite solution to 50 cc. of the sulphanilic acid solution.

Mix equal quantities of the urine and test solution and then add 1 or 2 cc. of strong ammonia, and shake. A positive reaction is indicated by bright red colored liquid and a salmon pink froth. Normal urine is colored yellow to orange and froth distinctly yellowish.

(i) Estimate the amount of *urea* by the hypobromite of soda method, using Doremus or larger ureometers. This estimation, like all quantitative











estimations, should be made on a sample from the mixed 12 or 24 hour amount. The hypobromite solution should be freshly made. For this purpose two laboratory stock solutions should be prepared, the bromine solution consisting of  $12\frac{1}{2}$  gms. each of bromine and sodium bromide, made up to 100 cc. with water; the alkaline solution of a 10% solution of sodium hydrate. Mix 10 cc. of the bromine solution with 25 cc. of the soda when required for use.

(j) Estimate the amount of *ammonia*.

This estimation is important in cases of acidosis, i.e. under conditions which lead to presence of diacetic acid and its precursors in the urine.

The following test is reasonably accurate. Measure 25 cc. of urine into a beaker and add 50 cc. of distilled water (free from ammonia) and .5 cc. of phenolphthalein solution. From a burette add decinormal sodic hydrate solution till a faint permanent pink tinge is established. Now add 5 cc. of formalin (neutralized if necessary by addition of decinormal soda). Owing to setting free of the acids previously in combination with ammonia the mixture becomes acid and it is necessary to add more decinormal soda till pink tinge again returns. Each 1 cc. of soda required for this final neutralization equals .0017 gramme of ammonia in the 25 cc. of urine used.

In routine urine analysis, apart from the reaction, the chemical tests commonly employed are those for

albumin and sugar. The other tests always should be made if indicated from clinical features of case, or from developments in the course of the routine analysis. Qualitative or quantitative tests may be required at times of uric acid, the purin bases, phosphates, chlorides, sulphates, pentoses, various drugs, and such like. For these a large work on urine analysis should be consulted.

### 3. *Microscopic examination.*

Urine which is turbid may be examined directly, but usually it is better with all samples to examine the sediment. This may be obtained by placing the urine in a conical glass and allowing to settle from 3 to 24 hours, or by use of centrifuge, the latter being preferable. The sediment is placed with a pipette upon a slide and examined first under the low power of the microscope, taking care to shut off most of the light. Later a cover glass may be applied and examination completed with the high power. With a large deposit a number of preparations should be made from different levels.

Amorphous urates may cover up other deposits and should be dissolved by warming the urine before centrifuging.

Much can be learned of the nature of a sediment by noting its color, general characters, the reaction of the urine, and whether in urine at the time of passing







from bladder. The student must early learn to differentiate between actual urinary deposits and extraneous particles which may gain entry, such as cotton, wool and linen fibres, mycelium of moulds, starch grains, vegetable cells and fibres.

The elements examined for microscopically or which may appear as sediments may be divided into three classes, (1) chemical, (2) anatomical, (3) bacterial.

1. The *chemical sediments* consist of various crystalline and amorphous chemical bodies which may exist normally or be present abnormally in urine. We may sub-divide them according to the reaction of urine in which found, color, and whether crystalline or amorphous.

(a) Sediments in acid urine:

Urates of soda and potash	amorphous, grayish pink
Uric acid,	crystalline, brick red.
Oxalate of lime,	crystalline, white.
Monocalcium phosphate,	crystalline (rare).
Acid ammonium urate,	crystalline.
Ammonia-magnesium phosphate,	crystalline, white.
Dicalcic phosphate (stellar),	crystalline, white.

Last three are found only in urine becoming alkaline and ammoniacal.

(b) Sediments in alkaline urine:

1. Alkalinity due to fixed alkalies;

(1) Basic phosphates of Ca. & Mg. amorphous, white.

Basic phosphate of Mg. crystalline, white.

Calcium carbonate, crystalline, white.

Dicalcic phosphate, crystalline, white.

(2) Alkalinity due to ammonia (usually fermentative);

Ammonium urate, crystalline, yellow.

Ammonio-magnesian phosphate, crystalline, white.

Basic phosphates of Ca. & Mg. amorphous, white.

Calcium carbonate.

Dicalcic phosphate.

Besides these sediments we have at times the rarer forms of chemical sediments, such as those of leucin and tyrosin, indigo, lime and magnesia soaps, cystin xanthin and hippuric acid.

It must be remembered that the presence of many of these chemical sediments does not necessarily mean an increase in their amount in the urine. The degree of concentration, the reaction and putrefactive changes determine the presence of many of them, so that in considering their import, these points must be taken into consideration.

2. The *anatomical sediments* consist of cellular elements derived from the urinary passages or from the blood. They consist of various forms of epithelium, tube casts, pus cells and blood. In all normal





urines a few epithelial cells, usually derived from the bladder, are found, but in urine from females vulval and vaginal epithelium is often abundant. As a rule, too, a few leucocytes (polymorphonuclear) can be seen. Apart from these constituents, the presence of other elements means some lesion of the urinary tract.

- (a) Epithelium may be derived from,
  - Kidney;
  - Renal pelvis and ureter;
  - Bladder and urethra;
  - (Vulva and vagina).

In differentiating between these cells, the size, shape and cell grouping must be carefully studied and other findings such as albuminuria or presence of pus or blood taken into consideration in drawing conclusions as to their import.

- (b) Tube casts are characteristic of lesions of the renal parenchyma. We find various forms of these casts depending on the intensity and nature of the lesion.

According to composition tube casts are described as epithelial, blood, leucocytic, finely and coarsely granular, fatty, waxy and hyaline. Cylindroids and mucous threads and cylinders must be differentiated from true tube casts.

- (c) Blood may be derived from any part of the urinary tract. Note must be taken of the presence

of clots, the nature of the admixture with urine and the presence of ureteral or true blood casts as well as other findings, in determining the source of the haemorrhage.

(*d*) Pus cells (leucocytes) are found in all irritative or inflammatory lesions of the urinary tract. Only rarely are they found alone as in rupture of an abscess into passages. In determining the site and character of the lesion we must consider the presence of albumin, epithelium or casts, as well as the general chemical and bacterial character of the urine.

3. Bacteria in the urine may be derived from the urinary passages or may enter the urine after its passage from the body (putrefaction).

The bacteria which may be found in affections of the urinary tract are as follows:

(*a*) The tubercle bacillus may be found in tuberculosis of any part of the urinary tract. It is examined for as described at page 51. It is always accompanied by small amounts of pus blood or epithelium.

(*b*) The gonococcus is not infrequently found in urine, not only in gonorrhoeal urethritis and cystitis but in "gleet" cases, where it is found in the so-called "gleet threads."

(*c*) Occasionally certain bacteria may be excreted through the kidney and multiply in the passages







leading to bacteriuria. This is seen at times in and after typhoid fever. Infections with *Bacillus coli* either via the kidney or by extension along urethra are fairly common, especially in women. The presence of calculi in the renal pelvis frequently determines the lodgment there of pyogenic bacteria. Either streptococci or staphylococci are found in the urine in pyaemic kidney.

(*d*) Bacteria may make their way to the bladder and upper urinary passages by being introduced on catheters or by direct extension along urethra, especially in females. These may be putrefactive bacteria alone or such pathogenic bacteria as *B. coli* or the ordinary pyogenic forms, while not infrequently several varieties of bacteria are present. Ammoniacal decomposition of the urine is a common result of such infection.

In urine analysis it is important to distinguish between bacterial decompositions of the urine in the bladder, and decomposition after its passage.

## SECTION II.

## BLOOD.

In ordinary blood examinations the points to be ascertained are the amount of haemoglobin and the number and characters of the red and white blood cells. Examination may also be directed toward the determination of the presence of malarial or other animal parasites or for presence of bacteria.

The blood may be obtained from the lobe of the ear, root of finger nail or the ball of the finger, the first being undoubtedly the best situation. The part should be washed with soap and water, then with a mixture of alcohol and ether, and rubbed dry before puncturing. The first drop of blood which exudes should be wiped away with sterile gauze. No pressure should be made to obtain the blood. A spear-headed needle or small lancet making a clean cut should be used for puncturing.

*Estimation of haemoglobin.* There are a number of instruments for this purpose, such as those of Fleischl, Dare and Gowers. The Tallqvist haemoglobin scale is much employed, and while not so accurate, especially in severe anaemias, has the advantage that the test is very quickly performed.

In the Fleischl instrument the blood collected in a special pipette is diluted with distilled water in a small cell, and its color compared with that of a colored bar viewed through a similar amount of water,





artificial light being used. This instrument is used in the following manner. After arranging the apparatus, cover the bottom of the outer cell with distilled water. Fill the capillary pipette with blood, taking care not to soil its exterior, and to have the tube just full. Rinse out the pipette in the water of the cell, and by dropping on more distilled water so as to fill the cell almost full. With the handle of the pipette, thoroughly mix the cell contents. This cell and the one over the colored bar are now carefully filled with water. Using a candle or an oil lamp (not daylight), compare the tint of the blood cell with that of the standard bar under the water cell, moving the bar till the two tints are the same. Read off the percentage of normal on the bar index.

With the Tallqvist haemoglobin scale, blood is absorbed into thin filter paper and the color contrasted (by daylight) with that of the scale. Full directions are furnished with each scale.

*Estimation of blood corpuscles.* The best instrument for this purpose is Thoma's haemocytometer. This has mixing pipettes for red and white cells and a counting chamber. The counting chambers may be marked off in various ways, the Zappert or Turk rulings being preferable. The blood must be diluted both for ease of counting and to prevent coagulation. For the red corpuscles Toison's dilution fluid is usually employed.

## Toison's dilution fluid,

Glycerine . . . . .	30 cc.
Sodium sulphate . . . . .	8 gms.
Sodium chloride . . . . .	1 "
Methyl violet . . . . .	.025 "
Distilled water . . . . .	160 cc.

For white corpuscle counting a fluid which will dissolve the red corpuscles is used, viz. .5% acetic acid solution. This may be tinged with methyl violet if desired.

*To count the red cells.* As a rule counts are made from blood diluted one hundred times, so that the red corpuscle pipette is filled up to mark 1 and at once dilution fluid is drawn in till mark 101 is reached. If a dilution of 1 in 200 is desired, draw in blood to mark .5 on stem and then fill up with the dilution fluid. Thoroughly mix the blood and the diluting fluid in the bulb of the pipette by twirling in fingers for a minute or two. After blowing out the fluid that fills the stem of the pipette, place a drop of the mixture from the bulb on the centre of the counting slide, and place over this at once a cover glass. The preparation if properly made should cover the central platform evenly, should not overflow the cell edges nor show air bubbles, and when examined should show an equal distribution of the corpuscles. Five minutes should be allowed for the corpuscles to settle on surface of platform. Examine under the microscope, using







high dry power and finding the ruled squares, count the red corpuscles on at least 100 squares. As each ruled square is  $1/4000$  cb. mm., each square measuring  $1/20 \times 1/20$  mm. and  $1/10$  mm. deep, we can readily calculate the number of corpuscles per cb. mm., by the following formula:

Number of corpuscles per 1 cb. mm.—

$$\frac{\text{No. of corpuscles} \times 4,000 \times 100 \text{ (dilution)}}{\text{No. of squares counted.}}$$

Normally the red blood cells number between 5,000,000 and 5,500,000 per cb. mm.

By this method of examination, information can also be obtained of the size and shape of the red corpuscles.

As the white corpuscles are tinged violet in using Toison's fluid as diluent, they are readily distinguished from the red corpuscles. The white corpuscles can readily be counted in such a preparation, at least 2,000 squares being counted, as can be done in the Zappert or Turk counting chambers by counting the sets of 400 squares bordering each side of the central set.

*To count the white cells.* While the white corpuscles can be counted as just recorded, it is preferable to estimate them separately. Using the white cell pipette, draw up blood to mark 1 on stem of pipette and draw in the acetic acid dilution fluid to

mark 11. Mix thoroughly and count as for red corpuscles. Normally the white cell count runs between 5,000 and 8,000 per cb. mm.

*Microscopic examination of blood films or smears.*

(a) Direct examination of moist films is called for, in determining poikilocytosis, leucocytosis, and in examining for malarial plasmodia, trypanosomes or filariae.

Moist preparations are made by touching the blood drop with a thoroughly clean cover glass, and then inverting this upon a slide. Only a small drop must be taken on the cover glass so that the corpuscle will spread out in a single layer on the slide. To prevent drying, the cover glass may be ringed with vaseline. Such films must be examined within a few minutes of preparation.

(b) Examination of films dried and stained is of much wider application, being employed to determine the same characters as the moist films, and in addition giving the forms and relative proportions of the white cells (for differential leucocyte count), and the staining characters of the red corpuscles. Blood smears should be spread so as to have the corpuscles in a single layer and uncrowded. Slides are best for this purpose, a drop being placed on surface of one slide and spread by edge of second held at an angle of  $45^\circ$  and drawn along first slide surface without pressure. By lessening the angle thinner smears are





obtained and thicker by increasing the angle. Thick smears are useful in study of trypanosomes and filaria. The smears are usually allowed to dry in the air. Fixation will depend on method to be employed in staining. Heat fixation gives best results in staining smears by Ehrlich's stain, with eosin and methylene blue, or haematoxylin and eosin. The smears should be placed in an oven held at  $110^{\circ}\text{C}$ , for one hour. As this method cannot always be carried out, a cheap and effective substitute is made by the use of a long triangle of brass, about .5 cm. in thickness. A gas or spirit lamp flame is applied to the apex, and after 5 or 10 minutes tests are made for the boiling point on the triangle. The slides are placed 2 to 3 cm. in front of this point, where the temperature averages between  $110^{\circ}$  and  $120^{\circ}\text{C}$ ., and are allowed to remain for 1 hour. A quicker method though less certain is to allow a few drops of 95% alcohol to flow over the smeared surface. Drain quickly on filter paper and light, and let alcohol burn itself out.

Absolute methyl alcohol for 2 minutes is a fairly satisfactory fixative. In using Wright's, Hasting's, Jenner's or Leishmann's stains, the methyl alcohol of the stain acts as fixative.

The composition of the common blood stains only will be considered.

(a) Wright's, Hasting's, Jenner's and Leishmann's stains are modifications of the Romanowski

method by which eosin and methylene blue are combined to get staining effects not seen when these stains are used separately, the action depending on the formation of one or more eosinates of methylene blue. The formula for Wright's stain is as follows:

Dissolve 1 gm. of methylene blue in 100 cc. of .5% aqueous solution of sodium bicarbonate. Place in a flask and steam for 1 hour in sterilizer. After cooling, add a 1-1000 aqueous solution of eosin (yellowish, water soluble) till a yellowish metallic scum forms on surface; usually about 500 cc. of the eosin solution will be required.

Filter, dry and collect the precipitate and dissolve it in pure methyl alcohol in the proportion of .3 gm. per 100 cc. This is then filtered and 25 cc. of methyl alcohol added to each 100 cc., making the permanent stain which should be kept well corked.

(b) Ehrlich's triple stain,

Saturated aqueous solution of acid fuchsin..	2.5 cc.
Distilled water . . . . .	2 "
Saturated aqueous solution orange G.....	6 "
Saturated aqueous sol., methylene green....	6.5 "

Add the methylene green slowly to others, constant'y stirring,

Absolute alcohol .. . . .	5 cc.
Distilled water . . . . .	10 "

More acid fuchsin may have to be added if methylene green tends to precipitate.

(c) Eosin and Methylene blue. In films fixed by heat the .5% alcoholic eosin in 70% alcohol is used as stain, while 1% aqueous eosin in distilled water seems best for films fixed in alcohol.







The methylene blue stains used are Loeffler's methylene blue or aqueous methylene blue.

(d) Haematoxylin and eosin.

Delafield's haematoxylin stain,

Haematoxylin crystals . . . . .	4 gms.
Absolute alcohol . . . . .	25 cc.
Saturated aqueous solution ammonia alum..	400 cc.
Mix these and let ripen in sun uncorked for from 2 to 3 weeks. Filter and add,	
Glycerine . . . . .	100 cc.
Methyl alcohol . . . . .	100 cc.

### *Staining of the Smears.*

(a) Wright's stain is used as follows: (1) Flood smear with the stain for 1 minute, covering same with watch glass to prevent evaporation. (2) Now add an equal amount of distilled water. A metallic scum forms on surface and stain becomes semi-translucent. Allow the stain to act from 4 to 5 minutes. (3) Wash thoroughly in water till film becomes pink, dry with blotting paper and examine.

With this stain red corpuscles are stained pink, nuclei shades of violet, eosinophile granules deep red, neutrophile granules pinkish violet, basophile granules blue, malarial parasites light blue, chromatin rose pink, platelets purplish.

In routine blood work this stain will be found a most satisfactory one.

(b) With Ehrlich's triple stain. The fixed films are covered with the stain for 6 to 8 minutes and are then washed in water, and dried. Care should be taken not to shake up the stain before using, and to withdraw the surface only for use, employing a pipette.

(d) Haematoxylin and Eosin. Place the fixed films in Delafield's haematoxylin stain for 3 to 5 minutes. Wash in tap water for two or three minutes and place in eosin 15 to 30 seconds. Wash in water, dry and mount.

This method gives clear nuclear staining, but apart from the eosinophile granules does not bring out the cell granules. Malarial and other parasites are only faintly stained.

*Differential leucocyte count.* For this count smears stained by one of preceding methods are required. At least 300 white cells should be counted and classified and percentage ratio estimated. The oil immersion objective will be required for the differentiation of the white cells in the majority of cases. On gaining experience the high or even low dry powers may suffice.

### *Bacteria in the Blood.*

In the circulating blood it is exceptional to have bacteria present in such number as to be demonstrable in smears, hence blood cultures must be employed for their detection. The blood is taken in a





sterile syringe (free from antiseptic) from one of the veins at bend of elbow, the part being prepared as for a simple surgical operation. The veins are made prominent by placing a ligature above elbow, tight enough to partially constrict the venous return.

At least 5 cc. of blood should be withdrawn and distributed in 3 tubes of sterile broth. One of the tubes on reaching the Laboratory may be poured into a flask containing about 100 cc. of broth so as to dilute and check action of the bacteriolytic substances of blood. The tubes and flask are then incubated for 24 hours at 37°C. and examined. Any bacteria which develop can be then isolated.

This procedure is valuable for determination of the causal bacterium of a septicaemia and as an additional diagnostic procedure in typhoid and paratyphoid infections or in ill-defined febrile disorders.

*Blood for Widal reaction and other serum tests.*

For the Widal reaction the blood can be collected as separate drops on a clean glass slide or on glazed paper and allowed to dry in the air. Care must be taken in collection that no antiseptic is mixed with the blood drop. If the serum only is required, the blood should be collected in Wright pipettes, the ends being carefully sealed in the flame. The same procedure serves for securing serum for determination of the opsonic index. The serum is best separated from the clot by use of the centrifuge.

For the Wassermann reaction about 5 cc. of blood should be withdrawn as in making blood culture and placed in a sterile test tube. If preferred, a number of Wright pipettes can be used and sufficient blood withdrawn to give 20 drops of serum. Half this amount will suffice for Noguchi's modification of this reaction.







## SECTION III.

## GASTRIC CONTENTS.

Examinations of the gastric contents are required to obtain information of the condition and digestive power of the gastric juice, and to determine the presence of any abnormal constituents, such as the products of fermentation. Accurate information, upon which conclusions can be based, is only to be obtained after several examinations and the use of test meals in a food free stomach, the contents being withdrawn at a set period.

The most commonly employed test-meal is the test breakfast of Ewald, and consists of 40 to 50 gms. ( $1\frac{1}{2}$  oz.) white bread and 300 cc. ( $\frac{1}{2}$  pint) of water or tea without milk or sugar. This is withdrawn one hour afterward through the stomach tube without the use of water. If water has to be used, measured small quantities only should be employed, but this seriously interferes with value of contents for test purposes. At the expiration of one hour, normally about 30 to 40 cc. of contents can be withdrawn, and in this we can demonstrate the presence of free and combined hydrochloric acid, pepsin, rennet, peptone and maltose.

Boas advises a special test meal in case of suspected carcinoma of stomach. It consists of from 350 to 400 cc. of very thin oatmeal gruel made with water

and salt, and given after lavage of the mouth and stomach. One hour afterwards the contents are expressed. The meal is given to prevent the introduction of lactic acid in the food, and the tests are made for lactic acid alone.

The examinations made of the gastric contents are physical, chemical and microscopical.

1. *Physical examination.*

(a) Measure the *amount* of contents obtained. More than 40 cc. indicates loss of motor and absorptive power of the stomach wall.

(b) Note the *color*, which is normally faintly yellow. The presence of blood, bile or much mucus, changes the color.

(c) Note the *odor*. If fatty acids, as acetic and butyric acids, are present, they can readily be detected in this manner.

(d) Note the amount of *mucus*. This is increased in nearly all forms of gastric affections, but more particularly so in "chronic" gastritis. The presence and nature of food fragments, portions of mucous membrane and the like, must also be noted.

2. *Chemical examination.*

In a chemical examination of the gastric contents the following reagents will be required:

Congo red solution. A 1 in 1000 aqueous solution of congo red powder.





Phloroglucin-vanillin solution of Gunzburg. Phloroglucin 2 gms., vanillin 1 gm., absolute alcohol 30 cc.

Dimethyl-amido-azo-benzol solution of Topfer. .5% alcoholic solution.

Phenolphthalein. .5% solution in 50% alcohol.

Alizarin. Saturated aqueous solution.

Uffelmann's reagent. Three drops of pure carbolic acid are dissolved in 10 cc. of water and 3 drops of 10% aqueous solution of the perchloride of iron added. The solution becomes a deep blue-black, and must be further diluted till an amethyst solution is obtained. This solution must be freshly prepared.

Decinormal sodium hydrate solution. Contains 4 gms. of NaOH in a litre of water. 1 cc. of the decinormal solution neutralizes .00365 gms. of HCl. This solution should be carefully titrated against normal or decinormal HCl and thus standardized.

The chemical tests are applied to the filtrate, one-half of the contents being filtered. For filtration washed cheese cloth or muslin is preferable to filter paper.

(a) Determine the *acidity* with litmus paper.

Acidity may be due to HCl free or combined (with albumins), to acid salts or to the presence of such fermentative acids as lactic, acetic or butyric.

(b) If acid, determine the *total acidity* by placing 5 cc. of the filtered contents in a capsule or test tube, adding 5 drops of phenolphthalein solution, and then carefully adding from a burette, decinormal sodic hydrate till completely neutralized. This will be indicated when a faint but definite pink tinge develops throughout. Normally from 40 to 60 cc. of the decinormal sodic hydrate solution are required to neutralize 100 cc. of the gastric contents.

(c) If acid, is the *acidity due to free acids* or acid salts? To determine this add a drop of the gastric contents to a drop of the congo red solution. If free acids are present a blue coloration appears. This is an azure blue with free HCl alone or HCl with lactic or other organic acids, while with lactic acid alone unless abundant the color is a dirty gray.

(d) If free acid is present, determine if *free HCl* exists.

(1) To a drop of the filtrate in a porcelain capsule add a drop of Gunzburg's phloroglucin-vanillin solution. Carefully evaporate high over the flame. If free HCl is present a rose red ring develops at margin of evaporating drop. This test will determine the presence of .05 parts or over of free HCl per mille.

(2) To a drop of the filtrate on a porcelain capsule, add a drop of Topfer's dimethyl-amido-azobenzol solution. This gives a red coloration, in the







presence of free HCl when present in .02 parts or over, per mille. It is a more delicate test than Gunzburg's and can be used to determine the amount of free HCl present.

(e) While in practice it is found that with normal amounts of free HCl, tests for *lactic acid* may be disregarded, yet when free acid is present a test should be made for lactic acid.

1. Place in a test tube about 10 cc. of Uffelmann's reagent. Holding the tube to light, add drop by drop 5 drops of filtered stomach contents. In the presence of lactic acid a lemon yellow cloud develops as each drop falls through the solution and the blue color is discharged.

This test is not always accurate as it is interfered with by excess of HCl, presence of acetic and butyric acids, alcohol or much glucose. A more satisfactory method is to shake up thoroughly 10 cc. of the filtrate with 50 cc. of ether, allow to stand for 30 minutes and remove the ether with a pipette. The ether is then carefully evaporated, the residue extracted with 10 cc. of water and used to make the test.

2. To a test tube of water add one or two drops of 10% solution of ferric chloride so as to give water a faint yellow tinge. Divide into two tubes and add to one 5 drops of the gastric contents. In the presence of lactic acid this tube will become distinctly yellow when compared to the control tube.

(f) The fatty acids, particularly *acetic and butyric acids*, are at times present as fermentative products. While the amounts of these acids usually rises and falls with the amount of the lactic acid, their odor is the best qualitative test. For chemical testing it will be necessary to extract them with ether in same manner as for lactic acid, and apply tests to the residue.

1. For acetic acid. Dissolve the residue in 2 cc. of water and neutralize with decinormal soda. Add a drop of 1% solution of ferric chloride, and if acetic acid is present a dark red coloration due to ferric acetate develops.

2. For butyric acid. Dissolve the residue in 2 cc. of water and add some calcium chloride (in substance). Butyric acid separates out and is readily recognized by its pungent rancid odor.

(g) To determine the amount of *free and combined* HCl in proportion to normal acidity.

1st. Determine the total acidity as per (b).

2nd. To 5 cc. of filtered gastric contents add 5 drops of the dimethyl-amido-azo-benzol solution. In presence of free HCl the solution becomes bright red. To this add decinormal soda, drop by drop, till the solution loses this bright red and alters to orange yellow. The amount of decinormal soda used as compared with that required for determination of total acidity gives relative proportion free HCl.





3rd. To 5 cc. of the filtered gastric contents and 5 drops of alizarin, now add the decinormal soda solution till the tube contents show a distinct purplish tinge. The difference between the amount of soda required to produce this reaction and that required for total acidity equals amount of soda required to neutralize the combined HCl.

A simple method for the determination of HCl acidity and total acidity is by use of a special tube known as the gastric acidimeter. The filtered gastric contents are run into the tube up to a certain mark and a few drops of combined Topfer's solution and phenolphthalein added as indicator. Decinormal soda is then added drop by drop and percentage read from tube graduations. Full directions accompany each tube.

(h) To determine the relative proportions of all the various constituents producing acidity the following method can be employed.

1. 10 cc. of the filtered contents are placed in a beaker and decinormal sodic hydrate solution is added, drop by drop, till a drop taken out with a small platinum loop, no longer gives a reaction with Topfer's solution. The reading on the burette gives the amount of solution required to neutralize the HCl (which has the strongest affinity for the alkali).

Now add a loop of the mixture to a drop of congo red solution. If this still indicates acidity, add more

of the soda solution till the congo red no longer indicates.

The reading on the burette gives the amount of the alkaline solution required to neutralize the free organic acids present.

A loop of the contents is next added to a drop of alizarin (indicates alkalinity), and if there is no violet reaction, add the soda solution till this develops. The reading on the burette gives the amount of the soda required to neutralize the acid salts.

If the contents are still acid to phenolphthalein, add more of the soda solution, till the rose-red color completely develops. The reading on the burette gives the amount of solution required to neutralize the combined HCl.

If there is no free HCl, nor free acid, titration can be commenced at the corresponding portion of the process just described.

(i) Test for *pepsin* by determining the digestive powers of the juice. In the absence of free HCl no pepsin will be found and tests must be directed towards its zymogen, pepsinogen.

Add a small shaving 1 cm. square and 1 mm. thick, of coagulated egg albumin to 10 cc. of the filtrate, and keep at 37°C, one to two hours. If there is no free HCl present, add 3 or 4 drops of a decinormal







solution of HCl, so as to transform any pepsinogen into pepsin. After two hours the shaving ought to be digested.

Quantitative estimation of the pepsin is carried out by making a series of dilutions and testing their digestive powers. This procedure is rarely called for in clinical practice.

(j) Test for *milk curdling ferment or rennet*, and its zymogen.

(1) In testing for rennet add 3 to 5 drops of the filtrate to 10 cc. of milk and keep at 37°C. If rennet is present the milk clots inside 15 minutes.

(2) If no coagulation occurs, test for the rennet zymogen, by neutralizing some of the gastric contents with fresh lime water, and then adding 3 to 5 drops, to the milk held at 37°C. when, if zymogen is present, coagulation occurs inside 15 minutes.

Quantitative tests for rennet or its zymogen can be carried out by a series of dilutions as is done for pepsin, but are rarely required clinically.

(k) Tests for the condition of the various food constituents of the test meal may also be carried out. Practically tests are required only for presence of propeptones, peptone, and the stage of starch digestion.

(1) Test for *propeptones* (or albumoses). Carefully neutralize 10 cc. of the filtrate with decinormal

soda. If any turbidity develops filter and to the filtrate add 1 drop of 10% acetic acid and an equal volume of saturated solution of sodium chloride and bring to a boil. If any turbidity or precipitate forms, again filter and add to filtrate a double volume of saturated solution of ammonium sulphate. This precipitates the primary and a large portion of the deutero-albumose.

2. Test for *peptone* by adding to the filtrate, after propeptones have been precipitated and filtered out, an equal quantity of caustic soda solution and a few drops of 1% solution of copper sulphate. A violet red color, the biuret reaction develops, if peptone is present.

3. The soluble or *dissolved starch* should be so far changed in the filtered gastric contents after the test meal as not to give either the reaction for starch or for erythro-dextrin. Test for these, by adding to several drops of the filtrate, a drop of weak solution of iodine (Gram's iodine diluted 6 times with water). Starch, if present, will give a blue reaction, erythro-dextrin, a purplish red.

### 3. *Microscopical examination.*

Portions of the unfiltered contents are examined under the microscope, with the low and high dry lenses and with the oil immersion lens. By this means we determine the nature of any food frag-





ments as muscle fibres, vegetable cells and fibres, starch granules, milk and fat globules; the presence of blood, pus, epithelium, and bacteria, including sarcinae and yeasts. In suspected cases of carcinoma search should be made for the Oppler-Boas bacillus which as a rule is present, though its presence is not distinctly characteristic of carcinoma. Mounting the material in weak iodine solution is often advantageous for differentiating between various elements of the deposit. Thus starch grains are stained blue, epithelial, blood and yeast cells yellow, while fat globules remain unstained.

Any fragments resembling portions of a new growth may be sectioned and examined in the usual manner.

#### *Examination of Vomitus.*

No systematic rules can be laid down for examination of vomited material, as findings will depend on character of food taken, time since taken, and extent of digestion apart altogether from presence of abnormal constituents.

Bile, blood and faecal matter are the most important abnormal constituents. The color of the contents usually is sufficient indication of presence of bile, while the odor suffices for faecal matter. Unaltered blood can usually be readily recognized by color or by finding corpuscles on microscopic exami-

nation. The gastric juice usually alters the color to dark brown or black as in the coffee ground vomit of certain cases of carcinoma. If corpuscles cannot be recognized, then one of tests for occult blood (page 143) should be made if blood is suspected.







## SECTION IV.

## FAECES.

The clinical features of case plus the results of inspection of the stools determine the lines to be followed in examination. The number of stools, their amount, form and consistence, color and odor must first be noted. Students should acquaint themselves with the microscopic appearances of commoner undigested food particles found in faeces such as vegetable and fruit cells and fibres, minute fragments of fibrous tissue and muscle. These, together with an enormous number of bacteria, fat droplets or crystals, much structureless debris and a few crystals of triple phosphate, make up the microscopic structure of the faeces.

The following constitute some of the commoner abnormal constituents for which examinations are made:

1. *Blood.* Fresh blood, especially if on surface of a formed stool is always from lower end of rectum. If streaked through a stool and bright in color from some part of colon, though if abundant may be from small bowel as in haemorrhage of typhoid fever. Usually when from stomach or small intestine the blood is intimately mixed with faeces and partially decomposed so that the stool is tarry. Small amounts

of blood may however not be apparent by any alteration in color, especially when from upper part of the gastro-intestinal tract, and tests for occult blood must then be employed. When blood is abundant a microscopic examination will generally show some unaltered corpuscles. The tests for occult blood should only be used on faeces when meat and meat juices have been excluded from the dietary for at least two days. The following tests are satisfactory:

(a) The benzidin test. The test solution is a saturated alcoholic solution of benzidin. About 3 cc. of this is mixed with 5 cc. of a thin emulsion in water of the faeces and then 3cc. of fresh peroxide of hydrogen and 3 to 5 drops of glacial acetic acid added, and whole well shaken. A deep bluish green color quickly develops in presence of blood pigment.

A more satisfactory method of applying this test is to first extract the blood pigment from the faeces. About 5 gms. of the faeces are emulsified in water and about one half volume ether added and well shaken and ether permitted to separate out when it is poured or pipetted off. This removes the fatty constituents of the stool. About 2 cc. of glacial acetic acid and same volume of ether as before are now added, again well shaken and ether allowed to separate out. Separation can be hastened by the use of the centrifuge. The acid ethereal extract is now poured off and used to make the test.









(b) The aloin test. The test solution is 3% solution of aloin in 70% alcohol, and must be freshly prepared. The test is applied to the acid ethereal extract prepared as just recorded. Equal amounts of the aloin solution and ethereal extract are mixed and 2 cc. of fresh hydrogen peroxide added. In the presence of blood pigment the mixture turns pink to cherry red. This reaction must develop within 5 minutes.

(c) The guaiac test. The test solution is freshly prepared by shaking together powdered gum guaiac and ether in a test tube and pouring off the ethereal extract after allowing solution to clear. This test is made with an acid ethereal extract of the stool, equal volumes of the extract, of the guaiac solution and peroxide of hydrogen being mixed. In the presence of blood a blue color quickly appears.

2. *Pus*. Leucocytes are absent from normal stools, hence the presence in any numbers of these cells is indicative of an acute inflammatory process in the intestine as acute dysentery, less often typhoid or tubercular ulceration of the bowel. Rupture of an appendiceal or other abscess may also account for its appearance. In dysentery the stool will contain abundant mucus and usually blood as well as pus.

3. *Mucus*. Small amounts of mucus adherent to surface of formed stools is a normal feature. Larger amounts either in liquid or formed stools indicates a

catarrhal condition of small or large intestine, definite masses being decidedly more abundant in lesions of large bowel. The mucus flakes and particles found in the diarrhoeal stools of tubercular enteritis, cholera or dysentery should be selected in examination for the causal organisms of these affections. In the condition known as mucous colitis, masses, long threads or even definite cylindrical moulds of mucus may be found, at times making up entire stools. Microscopically these are usually free from leucocytes but show desquamated epithelial cells, abundant bacteria and faecal particles and crystals.

4. *Gall stones and enteroliths.* Following an attack of gall stone colic the stools should be emulsified in water and strained through a fine sieve to determine if stone has passed into the intestine. Should there be any doubt as to nature of a concretion, powder a portion of it and extract with warmed alcohol and ether, equal parts. Pour off the alcohol-ether extract, allow to evaporate and examine residue for cholesterin crystals. A test can also be made for bile pigments by extracting the powdered concretion with normal hydrochloric acid and testing the extract with fuming nitric acid or iodine as in urine analysis.

5. *Intestinal parasites and their ova.*

(a) *Amoebae.* Amoebae in stools are not common in this country and infections by them, as amoebic







dysentery, are unknown except as brought in by those infected elsewhere. The fresh stool must be examined, mucous particles being selected, or in their absence a portion of emulsified stool. Specimens must be examined as moist preparations and should be kept at body heat, and diagnosis confirmed by noting change of shape of the amoebae. A drop of .5% aqueous solution of neutral red may be mixed with a drop of fluid faeces when being examined, the nucleus of the amoeba taking up this stain so that it becomes more readily visible.

Permanent preparations are often difficult to obtain as the amoebae disintegrate quickly when dry. Fairly satisfactory preparations may however be obtained from smears allowed to dry in the air and stained at once by Wright's blood stain; or by fixation of air dried smears in equal parts of absolute alcohol and ether plus 1% of a saturated alcoholic solution of bichloride of mercury, and staining with Loeffler's methylene blue.

Other protozoan parasites as *cercomonas* and *trichomonas intestinalis* and *balantidium coli* are found at times in examination of moist preparations from stools. Their pathological significance is uncertain.

(b) Intestinal worms and ova.

A careful inspection of stools will detect segments of tape worms or such worms as *ascaris lumbricoides* or *oxyuris vermicularis* if these are present. They

are more apt to be found after use of a purgative or of a vermifuge and purgative. The ova of the various intestinal worms require microscopic examination for their detection. The usual course is to emulsify portions of faeces and transferring drops to slide examine as moist preparations under the low and high dry objectives. A little practice soon enables one to pick out the ova with the low power objective, while the high power suffices for their differentiation. To isolate the smaller worms and concentrate the ova, 5 grammes of the faeces may be shaken up with 10 cc. of normal salt solution. The mixture is allowed to stand 15 minutes to settle or centrifuged for 2 minutes at about 250 revolutions. The salt solution is poured off and replaced by fresh and again shaken and allowed to settle. This may be repeated a third or fourth time till salt solution becomes fairly clear. In the deposit ova will be found if present, while smaller worms, such as *oxyuris vermicularis* or *necator americanus* are readily recognizable.

6. Bacteria of faeces will receive consideration in class-work in Bacteriology.





## SECTION V.

## EXUDATES AND TRANSUDATES.

Fluid effusions into the serous sacs may be serous, sero-fibrinous, haemorrhagic or purulent. The appearance of the fluid suffices for this differentiation.

A serous effusion may be either an exudate or transudate. An exudate usually has a specific gravity over 1018, while that of a transudate runs from 1005 to 1015. An exudate usually shows a high albumin content, averaging between 4 and 6%, while a transudate averages between 1 and 2.5%. Both may coagulate after removal, the exudate usually doing so. In *pleural fluids* the cell content is often of considerable diagnostic importance, tending not only to differentiate between exudates and transudates, but between exudates of different bacterial causation. The technique of examination for cell content (cyto-diagnosis) is as follows:

The fluid should be centrifuged immediately after removal before coagulation can occur. If not possible to do so at once, the fluid should be mixed with an equal bulk of 1.5% sodium citrate in normal salt solution till it can be centrifuged. Smears are made from the sediment in same manner as blood smears and stained by Wright's blood stain and examined. The character of cells present is noted and a differ-

ential count made. In transudates endothelial lining cells with scant numbers of lymphocytes are usually found. In tubercular exudates lymphocytes predominate, often making up 75 to 85% of nucleated cells present; while in exudates due to the pneumococcus or streptococcus polymorphonuclear leucocytes predominate, accompanied in the earlier stages by desquamated endothelial cells. The presence of considerable blood points to tubercular or malignant disease. In the latter case suspicious cell masses may be found in the sediment.

Examination for bacteria in serous exudates is frequently unsatisfactory. Pneumococci or streptococi may be found in recent effusions caused by them, but these bacteria soon die out unless lesion is progressive. They should be looked for in the sediment and cultures made therefrom. Tubercle bacilli are but rarely found by microscopic examination of the sediment in tubercular pleurisies, though their tubercular nature is demonstrable in most cases by injection of guinea pigs with the centrifuged sediment from 100 to 200 cc. of the effusion.

*Pericardial and peritoneal serous effusions* are examined similarly to pleural effusions. Caution must be observed in drawing any conclusions from the cell content. A turbid (milky) ascitic fluid may be caused by escape of chyle from the mesenteric lymph vessels.







*Hydrocele and spermatocele fluids* can be readily differentiated by the watery character of the latter and the presence of spermatozoa. In long standing hydroceles cholesterin crystals are very common. Less frequently cholesterin is found in old effusions in other serous sacs, and in cystic fluids.

*Fluid from cysts* may be tested for albumin and cell content. If pancreatic cyst is suspected the digestive action of the fluid on proteins and starch should be tried. For the former add a thin shaving of coagulated egg albumin to 10 cc. of the fluid and keep at 37°C. for one hour, noting digestion. For the latter mix equal parts of the fluid and a very thin, freshly made boiled starch solution, and keep at 37°C. If amylolytic ferment is present a drop of solution will gradually cease to strike blue when a drop of weak iodine solution is added, while after one hour dextrose can be detected by Fehling's test.

In a suspected hydatid cyst, examination should be made of sediment for hooklets.

#### *Cerebro-spinal fluid.*

This fluid is usually obtained by lumbar puncture and affords valuable diagnostic information in various forms of meningitis, in paresis and tabes.

In withdrawing the fluid, note must be made of rate of flow as giving some indication of the intracranial pressure. Usually 10 cc. can readily be obtained and not uncommonly 30 to 50 cc.

Normal fluid, and that from cases of paresis and tabes, the majority of cases of tubercular meningitis and brain tumors is clear and watery; while the fluid is cloudy to purulent, in all forms of acute meningitis. With cloudy or turbid fluids, smears are made directly from the fluid or from the sediment. These should be stained by Loeffler's methylene blue and also by Gram's method to demonstrate bacteria present and character of cellular exudate. This usually suffices for bacteriological diagnosis, but if thought necessary to obtain cultures, 3 to 5 drops of the fresh fluid should be at once added to broth and incubated at 37°C. Subcultures can be made later on blood serum or egg media.

If fluid is suspected to be from a case of tubercular meningitis it should be centrifuged and sediment stained by Ziehl-Neelsen method. Examination should also be made for globulin increase and cell content, the latter being determined from the smears stained for tubercle bacilli or by examination of sediment as in pleural fluid. The globulin and cell numbers can be determined as described later.

Cerebro-spinal fluid in suspected paresis or tabes should be examined for globulin increase, lymphocyte (cell) count and for Wassermann reaction.

1. Tests for globulin. These tests should be employed only on fluid entirely free from blood.





(a) Ross-Jones ammonium sulphate test. In a small test tube, place 2 cc. of saturated aqueous solution of ammonium sulphate. Carefully add 1 cc. of the fluid so as to overlies this. At the line of juncture there is with normal fluid no contact ring or but the faintest opalescence, while a distinct white ring forms with increase in globulin.

(b) Noguchi's butyric acid test. To .1 cc. of the fluid add .5 cc. of a 10% solution of butyric acid in normal salt solution and bring to a boil. Add now .1 cc. of decinormal sodic hydrate and again raise to boiling point. With normal fluid there develops only a faint turbidity, while with increased globulin content a flocculent precipitate appears, the amount and rapidity of its formation depending on amount of increase. The tubes should be examined at end of 15 minutes, 1 and 2 hours, the longer period being required when increase is slight only.

## 2. The lymphocyte count.

This is made by drawing 2% aqueous solution of methylene blue into white corpuscle pipette of haemocytometer up to mark .5 and then filling up pipette with the cerebro-spinal fluid. After admixture a drop is placed on the platform of the Rosenthal counting chamber and cells counted with high dry objective.

The Rosenthal counting chamber is ruled in squares each equal in superficial area to 25 squares

of the Zappert or Turk counting chamber of the haemacytometer, while the depth is double that of these chambers. A count of 80 squares equal 1 cb. mm. and this number should be least number counted.

Normally not more than 8 lymphocytes are found, while in paresis the count may run from 12 to 60. A lymphocytosis is also found in tubercular meningitis and in some cases of syphilitic meningitis.

3. The Wassermann reaction or Noguchi's modification. At least 1 cc. of the cerebro-spinal fluid should be used in making these tests. (See page 78).







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